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(Evelyn Rosario)

LUD-5253.5 DIV (09885911)

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PATENT & TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:
Thierry BOON-FALLEUR et al..

Group Art 1644

Serial No. 08/819,669

Examiner: P. Gambel

Filing Date: March 17, 1997

For: TUMOR REJECTION ANTIGEN PRECURSORS, TUMOR REJECTION ANTIGENS AND USES THEREOF

REPLY BRIEF

(37 CFR § 1.193(b))

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Pursuant to 37 CFR §1.193(b), Appellants now submit this Reply Brief, in response to the Examiner's Answer of September 17, 2004

This Reply Brief is filed within the two month period provided for by 37 CFR §1.193(b)(1). Three copies are provided.

No fee is believed due in connection with the submission of this Reply Brief, but if this is not correct, authorization is hereby given to charge such fees to Deposit Account 500624.

I. GROUPING OF CLAIMS

At page 3 of the Examiner's Answer, the Examiner states, correctly, that Appellants had grouped claims 183, 185, 186, 188, 189 & 191 (not a second 189 as reported by the Examiner in a first group), and then claims 184, 187 & 190 in a second group.

The Examiner, however, then exceeds his authority and has taken claims 189-191 and placed them in a third group.

There is no provision for this type of action on the Examiner's part. According to MPEP 1208, page 1200-18, the Examiner's Answer must state:

> "(W)hether the Examiner disagrees with any statement in the brief that certain claims do not stand or fall together, and, if the Examiner disagrees, an explanation as to why those claims are not separately patentable."

Appellants did not argue claims 189-191 separately. Further, in his Examiner's Answer, the Examiner cites a new reference, and raises issues against claims 189-191 not raised previously, in clear contravention of 37 CFR § 1.193(a)(2). While the Examiner has not called his treatment of claims 189-191 a New Grounds of Rejection, he is invited to show where, in the course of prosecution he has applied reference "k," i.e., Illustrated Dictionary of Immunology, or has raised a rejection to claims 189-191, such as that set forth at pages 38 et. seq. of the Answer.

Notwithstanding these procedural irregularities, given the seven year pendency of this application, and Appellants' desire to expedite resolution of this case as quickly as possible, Appellants will agree to the grouping of claims 189-191 together, as a separate group, and will address the Examiner's new arguments. They do ask the Board to comment on this procedure, however, because it appears to Appellants, as noted supra, that this is highly irregular.

II. ART OF RECORD

Appellants note that at page 4, the Examiner lists 11 references, and refers to these as "PRIOR ART."

The only reference of the eleven which is prior art is reference "B," i.e., Boon, et al., Cancer Cells 1: 25-28 (1989).

It is also noted that, while the Examiner refers to eleven references in his Answer, a review of the Answer indicates that Ding, et al, Biochem Biophys Commun. 202: 549-555 (1994) (reference F), is not cited or relied upon. As this reference is of record, however, Appellants will refer to it, as is their right.

As to the property of the other references as valid citations, this will be discussed infra.

III. REPLY TO THE EXAMINER'S ANSWER WITH RESPECT TO THE WRITTEN DESCRIPTION REJECTION

The "crux" of the Examiner's position on this issue appears to be set forthover pages 9-10 of his Answer, to which attention is directed.

These assertions, however, are not true. Further, they ignore other features of the invention, which are discussed in the application and were discussed in Appellants' Brief on Appeal.

MAGE-5 is presented as SEQ. ID NO: 16. It is labeled as genomic DNA, but within nucleotides 645-908, one finds the sequence presented in triplets, i.e., a coding region. One of ordinary skill in the art recognizes such as the molecule that constitutes cDNA, because cDNA contains only coding regions.

This is also true for MAGE-51 (SEQ. ID. NO: 17), at nucleotides 645-992 SEQ. ID. NO: 18, for MAGE-6, is cDNA, SEQ. ID. NO: 19, for MAGE-7, presents a coding region at positions 686-937. SEQ. ID. NO: 20, for MAGE-8, does the same, starting at position 452 and ending at position 1156. SEQ. ID. NO: 21, for MAGE-9, presents a coding region at positions 428-1375. SEQ. ID. NO: 22, for MAGE-10, presents a coding region at positions 334-920, and SEQ. ID. NO: 23, for MAGE-11, does so at position 601-1107.

Given the rules of coding degeneracy, the amino acid sequence for these molecules has been given. The triplet coding "ATG" for example, is always methionine. One can readily and easily present an amino acid sequence, given the nature of the genetic code.

The Examiner goes on to argue that there is no evidence that MAGE 4-11 stimulate CTLs, or that CTLs are available to test the properties thereof.

In response, it is noted that the Examiner has in fact conceded homology of the sequences Homologous nucleotide sequences can be expected to encode homologous proteins, which in turn behave homologously. Indeed, Appellants have provided a thorough listing of references which evidence the fact that the MAGE molecules do stimulate T cells.

With respect to Appellants' alleged failure to provide specific tumor rejection antigens other than SEQ. ID. NO: 26, it is submitted that this is not relevant, as the claims do not recite specific TRAs. Yet further, U.S. Patent No. 5,405,940, which is of record, shows the regions of the various MAGE coding sequences which are comparable to SEQ. ID. NO: 26, and established that the peptides encoded thereby do function as TRAs.

With respect to the statements at page 10 of the Examiner's Answer, the Examiner asserts that "the appropriate CTLs" had to be "readily available" and one must know "the relevant class I HLA molecule associated with each MAGE TRAP protein." Again, neither statement is true.

Taking the second statement first, the evidence supplied with Appellants' Brief shows that the TRAPs yield peptides which complex with different HLA molecules. There is not a

"one on one" correspondence of HLA and TRAP. With respect to the need for CTLs, again the Examiner appears to be assuming that purified CTLs are necessary. In act, all one needs is a blood sample. The relevant CTLs are developed therefrom.

The Examiner appears to rest his case extensively on the disparities in non-prior art De Plaen et al., because MAGE-7 acts in a way different from the others.

The relevancy of this to a written description rejection is not clear. There isn't a specific claim to MAGE-7 presented. Further, if MAGE-7 does not posses the relevant functional attributes of the claims, then it is not being claimed.

At page 11, the Examiner appears to be resting a rejection on the absence of complete hybridization conditions in the claims.

Appellants would like to point out, however, that, following a rejection made in September 26, 2000 (page 26), Appellants presented an amendment whereby the present conditions were added. See the amendments of October 6 and November 13, 2000.

In paper number 37, the Examiner withdrew the rejection based upon the absence of the actual stringent conditions. That rejection was made under 35 USC § 112, second paragraph. The Examiner never posited a rejection under 35 USC § 112, first paragraph because of an allegedly incomplete set of stringency conditions.

The fact is, all of the nucleic acid molecules described in the specification do hybridize to the referenced SEQ. ID. NO: 8 at the recited conditions. With the exception of MAGE-7, all according to De Plaen et al. appear to function in the same way. Indeed, the Ding reference, previously relied upon by the Examiner but now apparently dropped, shows yet further molecules which hybridize to SEQ. ID. NO: 8, and function as TRAPs. The authors of Ding so state. Hence, the Examiner's conclusions are without bases and do not support his position.

The Examiner then goes on to rely on five Federal Circuit decisions, i.e., Vas-Cath Inc. v. Mahurkar, 19 USPQ 2d 1116 (Fed. Cir. 1998); Fiers v. Revel, 25 USPQ 2d 1601 (Fed. Cir. 1993); Amgen Inc. v. Chugai Pharmaceutical Co. Ltd., 18 USPQ 2d 1016 (Fed. Cir. 1987); Fiddes v. Baird, 30 USPQ 2d 1481; Regents of The University of California v. Eli Lilly & Company, 43 USPQ 2d 1398 (Fed. Cir. 1997). These cases have been relied upon previously, and Appellants have presented arguments as to why they are not pertinent to the present case. The Examiner has not addressed these arguments other than to deem them "unpersuasive." As such, Appellants will again set forth their arguments.

The Vas-Cath court, after discussing an alleged lack of clarity in the area of the law, stated:

> "Although [the applicant] does not have to describe exactly the subject matter claimed, . . . the description must clearly allow persons of ordinary skill in the art to recognize that [he or she] invented what is claimed."

Vas-Cath at 1116. The claimed subject matter is set forth in terms that one of ordinary skill could readily and clearly understand. A reference sequence is presented, as are precise standards of hybridization. Hybridization of nucleic acid molecules to each other is a standard technique, and it is well recognized that as conditions become more stringent, the number of hybridizing sequences will decrease. The specification also describes how to determine if a molecule which meets the standards of hybridization also satisfies the functional requirements of a tumor rejection antigen precursor. As such, the Vas-Cath standard is satisfied.

Fiers v. Sugano, 23 USPQ 2d 1601 (Fed. Cir. 1993), is inapposite to the present application. Vas-Cath stressed the fact based nature of any inquiry as to whether the written description requirement is satisfied.

In the Fiers case, the claimed invention was isolated DNA molecules – with no disclosure of the molecules claimed. The Appellants, in Fiers, were attempting to claim these DNA

molecules on the basis of the disclosure of a protein, and a method for isolating DNA; however, no DNA was in fact isolated.

In the present case, proteins are claimed. As has been pointed out, supra, the sequence of proteins is defined by the exons of the nucleic acid molecules encoding them. These are provided in this disclosure. This is not a case "on all fours" with or related to Fiers, where, as was pointed out, supra, there wasn't a single molecule disclosed which satisfied the claims.

Amgen Inc. v. Chugai Pharmaceutical Co., 18 USPQ 2d 1016 (Fed. Cir. 1991), IS TO THE SAME END. In Amgen, the claims held not to satisfy the written description requirement were defined totally in functional terms. No structural parameters whatsoever were recited. Indeed, in terms of the claims at issue in Amgen, the Federal Circuit looked to the disclosure of

> "a few EPO analog genes"

and stated:

> "This 'disclosure' might well justify a generic claim encompassing these and similar analogs, but it represents inadequate support for Amgen's desire to claim all EPO analogs."

As has been pointed out, supra and in Appellants' main brief, a number of molecules representative of the claimed genus are disclosed, both in terms of structural similarity (i.e., the ability to hybridize to a reference molecule) and functional similarity (i.e., the ability to function as TRAPs). Appellants are not claiming all TRAPs generically. They are claiming those TRAPs which meet specified criteria set forth in the claims, and disclose a good number of species which meet these criteria. As such, Amgen is not seen as relevant either.

The Examiner's statement of the facts in Fiddes v. Baird, 30 USPQ 2d 1481 (Fed. Cir. 1994), is somewhat misleading. The Federal Circuit pointed out:

> "For bovine pituitary FGF the patent teaches both the amino acid sequence and a theoretical DNA sequence for the factor."

Fiddes at 1483. It was uncontested that no DNA sequences whatsoever were disclosed, and DNA was being claimed. The Court actually heed that there was no written description, for any nucleic acid molecule.

The Examiner goes on to refer to "The Guidelines for The Examination of Patent Applications Tender The 35 USC § 112, First Paragraph Written Description Requirement." Indeed, the Examiner has referred thereto, each time an office action has issued. In response, Appellants have drawn the Examiner's attention – and now respectfully refer the Board of Appeals – to example 9 of those Guidelines. For convenience, a copy is attached hereto.

In contrast to the example given in the guidelines, Appellants describe a number of species which fit within the structural confines of the claim. A specific activity is described, i.e., that molecules which satisfy the structural characteristics of hybridization funciton as TRAPs. The evidence of record indicates that this is in fact the case. The Examiner rests his position on an argument that one molecule, i.e., MAGE-7, which does satisfy the structural confines of the claim, has not been shown to share the function.

It is submitted that it is a rare case where every molecule which falls within the scope of a claim functions as described. This is an enablement question, rather than one of written description, however, and it will be discussed in the context of the Lack of Enablement rejection, infra. In any event, the Guidelines expressly referred to by the Examiner clearly indicate that one may only need to disclose a single species within a genus to satisfy the written description requirement. Appellants have gone far beyond this. The USPTO's own internal guidelines support Appellant's position, which is that the claims satisfy the Written Description requirement.

Finally, the Examiner cites to University of California v. Eli Lilly & Co., 43 USPQ 2d 1398, 1406 (Fed. Cir. 1997), as allegedly holding that:

> "(T)he description of a genus is achieved by the recitation of a representative number of DNA molecules, defined by nucleotide sequence, falling within the scoping that genus."

The University of California case, however, did not so hold. What the Court stated is that:

> "In claims to genetic material, however, a generic statement such as 'vertebrate insulin cDNA' or 'mammalian insulin cDNA' without more, is not an adequate written description of the genus because it does not distinguish the claimed genus from others, except by function."

The claims at issue recited, e.g.:

> "A DNA transfer vector comprising an inserted cDNA consisting essentially of a deoxynucleotide sequence coding for human proinsulin, the plus strand of said DNA having a defined 5' end, said 5' end being the first deoxynucleootide of the sequence coding for human proinsulin."

As the decision also makes clear, the patentees had no sequences described which satisfied this claim.

On its face, the University of California case does not control. First, the claims on appeal do not recite function only. Hybridization language, as is recited, is structural language. Second, in contrast to the facts in University of California, Appellants do describe a number of sequences that satisfy the language of the claim.

Further, the USPTO has clearly indicated that claims such as those presented in the subject application are to be treated differently than those of the type at issue in the University of

California cse. Example 7 of the Interim Written Description Guidelines, referred to by the Examiner, discusses claims in the format:

An isolated DNA comprising SEQ. ID. NO: 16.

The commentary indicates that the University of California case is controlling, and the written description requirement is not satisfied.

The Guidelines go on, however, in Example 9, to describe the following claim:

> An isolated nucleic acid that specifically hybridizes under highly stringent conditions to the complement of the sequence set forth in SEQ. ID. NO: 1, wherein said nucleic acid encodes a protein that binds to a dopamine receptor and stimulates adenylate cyclase activity.

The hypothetical states that hybridization conditions are recited (6XSSC, 65°C – conditions LESS descriptive than what is recited in the current claims), and that only a single sequence is described.

Nonetheless, the written description requirement was deemed satisfied, and the University of California, case was not referred to at all.

It is submitted that the reliance on these cases by the Examiner is inappropriate, as they do not support his position.

Following the relevance on the cases, supra, the Examiner raises, for the first time, an issue relating to vaccines, and an alleged low immunogenicity and low immune response.

The fact is, however, that TRAPs which the Examiner admits fall within the scope of the claim and which the Examiner admits are adequately described do function as vaccines. Consider, for example, U.S. Patent No. 6,565,857 claim 10 in particular, as well as the attached

reference. These were not made of record previously because, as pointed out herein, this is the first time the Examiner has raised this issue. Attached are Vantomme, et al, J. Immunother 27 : 124-135 (2004); Zhang, et al, J. Immunol 171 : 219-225 (2003); Atanackovic, et al, J. Immunol 172 : 3289-3296 (2004); Kruit, et al, Ahstract 1882 (page 470) ASCO Proceedings 2000.

The Examiner goes on to present a discussion of non-prior art references which, it is submitted, is not relevant. The claims as set forth are product claims. There are no use claims presented. Further, in terms of what "was difficult at the time the invention was made." It is pointed out that once Appellants provided their definition of a TRAP, and protocols for securing these, such difficulty was obviated, at least with respect to TRAPs – and that is all that is at issue here.

Following the Examiner's discussion of non-prior art references, he again refers the reader to the Guidelines for the Examination of Patent Applications Under The 35 USC § 112, section 1 'Written Description' requirement. As has been pointed out, supra, however, the Guidelines have been considered, and are believed to support Appellants' position. Example 9, for example, has been brought to the Examiner's attention more than once, and has not been addressed by the Examiner.

Appellants have attempted to follow the Examiner's "Response To Arguments," point 11, at page 26 et seq. Most of these points have been addressed already, however, several are embedded herein, for the first time.

The Examiner harps on the error in SEQ. ID. NOS: 7 & 8, as evidence that "possession of a correct sequence was not necessarily readily apparent."

In response, Appellants note that correction was permitted. If written description were not presented for these sequences, then why was the correction permitted? If the Examiner's position were correct, then the correction would not have been proper. Further, the Examiner does not seem to dispute that a nucleic acid molecule which differs from SEQ. ID. NO: 8 or

SEQ. ID. NO: 7 by one nucleotide would hybridize to it. And indeed, such nucleic acid molecules are TRAPs.

Finally, Appellants find themselves asking the following rhetorical question: if the Examiner feels that written description is not satisfied because of a lack of evidence for functionality generally, then why does he accept that SEQ. ID. NOS: 7 & 8 per se all adequately described the evidence for these parallels the evidence for all other a sequences claimed. It is not understood why it is deemed satisfactory for these, but not the others.

* * *

The Examiner then includes a "Response To Argument" section (point 11, starting at page 26 of his Answer) where he first attempts to show that Appellants' arguments regarding the impropriety of using non-prior art references. The Examiner quotes In re Koller, 204 USPQ 702 (1980), quoting footnote 5, which is at page 706 (not 707).

The Examiner has truncated the footnote, however, and has deleted the ending portion thereof, which states:

> "Whatever might have been said enroute to decision in these cases, the fact situation in none of them establishes a precedent for using a later existing state of the art in determining enablement under 35 USC §112

(emphasis added). As Appellants have pointed out previously, Koller extended this, in holding:

> "In Hogan, an analysis using later filed references to determine the scope of enablement was found to be impermissible. Similarly, it cannot be allowed when, as here, the description requirement is in issue."

Koller at 707. Nothing the Examiner has cited to or applied evidences what the state of the art was in 1992. Science advances. Many of the techniques employed in the references used by the Examiner were not even available or readily known at the time the application was filed. Appellants maintain that the Examiner's reliance on these references cannot be sustained.

With respect to the Examiner's response to Appellants' arguments regarding Written Description, it appears that the Examiner confuses enablement with written description, as the thrust of his argument appearing to be that MAGE-7, which the Examiner concedes meet the structural criteria of the claims, does not function as a TRAP. However, this is an enablement issue, not one of written description.

No rule or regulation or case precedent requires a complete sequence of any and every molecule, protein or amino acid, that falls within a claim. The '"Guidelines" referred to supra, make this clear. In deciding the University of California case, the Federal Circuit expressly declined to issue any holding on this issue and the Guidelines followed thereafter. One must assume, then, that the USPTO did accept that claims of this type presented herein satisfy the written description requirement. Appellants have provided a precise definition of what is claimed, rather than the alleged "mere wish or plan for obtaining the claimed invention." As has been pointed out, repeatedly, the Examiner's arguments do not address the issues or the claimed subject matter and ignore the relevant law. As such, the rejection should be reversed.

IV. RESPONSE TO THE EXAMINER'S ARGUMENT THAT CLAIMS 184, 187 AND 190 DO NOT SATISFY THE WRITTEN DESCRIPTION REQUIREMENT

Claims 184, 187 and 190 are dependent claims. The ultimately depend from claim 183. Hence all arguments presented previously are applicable.

These three claims all require that the claimed molecule include the amino acid sequence of SEQ. ID. No: 26. SEQ. ID. NO: 26 is a tumor rejection antigen. It is disclosed as such in the specification, and as has been noted, the USPTO has granted a patent on this molecule.

The Examiner's position here is difficult to follow, because the Examiner concedes that SEQ. ID. NO: 26 is described as a peptide which complexes with HLA-A1, and stimulates CTLs. See page 36 of the Examiner's Answer, penultimate paragraph. (The fact that the peptide can form a complex with another HLA molecule is not seen as relevant, since the claims do not require a specific HLA molecule as a partner).

Nor is the Examiner's position that it was necessary to have appropriate CTLs available a viable argument. Appellants explain what the peptide of SEQ. ID. NO: 1 will complex with HLA-A1, and the resulting complex will stimulate the formation on of CTLs. Assuming arguendo that no CTLs were available, surely it is within the skill of the artisan to make a nonamer, admix it with a cell presenting HLA-A1 molecules, and then stimulate the production of the CTLs. Once these are in hand, the CTLs can identify relevant other complexes, i.e., those formed by action of TRAPs containing SEQ. ID. NO: 26.

The Examiner repeats the same arguments at page 38 that he has made several times in the Answer. The arguments do not become any more persuasive with repetition. The rejection should be reversed.

V. THE REJECTION OF NEW GROUP CONTAINING CLAIMS 189-191

As was pointed out, supra, Appellants did not group claims 189-191. The Examiner improperly did so for the first time in his Answer. Nonetheless, in an effort to expedite the 7 years of prosecution, Appellants have accepted this grouping rather than request remand, but do ask the Board to address the propriety of the Examiner's Answer.

With respect to the Examiner's position here, the Examiner concedes that MAGE-1 itself provides data that supports the criteria set forth by the Examiner as necessary for a vaccine. Appellants have submitted evidence herewith to show that other MAGE TRAPs are useful as vaccines. While it is agreed that this is late filed, Appellants have submitted it immediately following the first time the Examiner raised the issue.

It is believed that the arguments advanced supra, taken with the supporting evidence lead to the conclusion that the rejection of claims 189-191 for failing to satisfy the Written Description requirement should be reversed.

VI. THE EXAMINER'S POSITION WITH RESPECT TO THE ENABLEMENT OF CLAIMS 183, 185, 186, 188, 189 AND 190 (Sic; Not 198 As Set Forth At Page 41 Of The Examiner's Answer)

The Examiner sets forth his position at page 41 et seq. In large part, this discussion is a word for word copy of the Examiner's position vis a vis written description.

Appellants recognize, however, that the Courts have made clear that the enablement and written description requirements are separate and distinct requirements. The underlying facts of the application, however, are the same as for written description. Hence, Appellants will not belabor the Board with a repetition of their argument supra. The fact is, nucleic acid molecules were isolated. Coding regions were presented. In their Brief on Appeal, Appellants discussed the relevant law – which the Examiner has not addressed – and explained how the non-prior art relied upon by the Examiner was not relevant. Appellants point to Sections C and D (pages 15-22), of their main brief.

The Examiner has not addressed any of these arguments in his Answer, other than to state they are not persuasive.

How can one respond to an argument if no argument is made? Indeed, it is submitted that the Examiner has not made out even a prima facie case for each of , enablement, and Appellants' arguments presented previously remain controlling. The rejection under 35 USC §112, first paragraph, arguing each of enablement, should be reversed.

VII. THE NEW REJECTION OF CLAIMS 189-191 AS LACKING ENABLEMENT

Appellant are compelled to address this new rejection as they do not acquiesce in it.

As was pointed out, supra, the Examiner has conceded that MAGE-1 satisfies his criteria for a vaccine. Again, with apologies for submission of the late reference, necessitated by the Examiner's newly stated position, there is additional evidence to support Appellants' position. A prima facie case for lack of enablement has not been made out and the rejection should be reversed.

VIII. CONCLUSION

While the Examiner's 55 page Answer is hefty, it does not address Appellants' position on the rejections, let alone refute it. Hence, it is respectfully submitted that all rejections, both old and new, under 35 USC §112, first paragraph, should be reversed.

Respectfully submitted,

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e.g. expression vectors, the necessary common attribute is the ORF (SEQ ID NO: 2).

Weighing all factors including (1) that the full length ORF (SEQ ID NO: 2) is disclosed and (2) that any substantial variability within the genus arises due to addition of elements that are not part of the inventor's particular contribution, taken in view of the level of knowledge and skill in the art, one skilled in the art would recognize from the disclosure that the applicant was in possession of the genus of DNAs that comprise SEQ ID NO: 2.

Conclusion: The written description requirement is satisfied.

Example 9: Hybridization

Specification: The specification discloses a single cDNA (SEQ ID NO:1) which encodes a protein that binds to a dopamine receptor and stimulates adenylate cyclase activity. The specification includes an example wherein the complement of SEQ ID NO: 1 was used under highly stringent hybridization conditions (6XSSC and 65 degrees Celsius) for the isolation of nucleic acids that encode proteins that bind to dopamine receptor and stimulate adenylate cyclase activity. The hybridizing nucleic acids were not sequenced. They were expressed and several were shown to encode proteins that bind to a dopamine receptor and stimulate adenylate cyclase activity. These sequences may or may not be the same as SEQ ID NO: 1.

Claim:

An isolated nucleic acid that specifically hybridizes under highly stringent conditions to the complement of the sequence set forth in SEQ ID NO: 1,

wherein said nucleic acid encodes a protein that binds to a dopamine receptor and stimulates adenylate cyclase activity.

Analysis:

A review of the full content of the specification indicates that the essential feature of the claimed invention is the isolated nucleic acid that hybridizes to SEQ ID NO: 1 under highly stringent conditions and encodes a protein with a specific function. The art indicates that hybridization techniques using a known DNA as a probe under highly stringent conditions were conventional in the art at the time of filing.

The claim is drawn to a genus of nucleic acids all of which must hybridize with SEQ ID NO: 1 and must encode a protein with a specific activity.

The search of the prior art indicates that SEQ ID NO: 1 is novel and unobvious.

There is a single species disclosed (a molecule consisting of SEQ ID NO: 1) that is within the scope of the claimed genus.

There is actual reduction to practice of the disclosed species.

Now turning to the genus analysis, a person of skill in the art would not expect substantial variation among species encompassed within the scope of the claims because the highly stringent hybridization conditions set forth in the claim yield structurally similar DNAs. Thus, a representative number of species is disclosed, since highly stringent hybridization conditions in combination with the coding function of DNA and the level of

skill and knowledge in the art are adequate to determine that applicant was in possession of the claimed invention.

Conclusion: The claimed invention is adequately described.

Immunologic Analysis of a Phase I/II Study of Vaccination with MAGE-3 Protein Combined with the AS02B Adjuvant in Patients with MAGE-3-Positive Tumors

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Abstract: In a phase I/II study, patients with solid metastatic MAGE-3-positive tumors, mainly melanoma, were vaccinated with recombinant MAGE-3 protein combined with the immunologic adjuvant AS02B comprised of MPL and QS21 in an oil-in-water emulsion. The recombinant MAGE-3 protein was made up of a partial sequence of the protein D (ProtD) antigen of *Haemophilus influenzae* fused to the MAGE-3 sequence. The vaccine was given intramuscularly at 3-week intervals. Patients whose tumors stabilized or regressed after 4 vaccinations received 2 additional vaccinations at 6-week intervals. MAGE-3 and ProtD antibody and cellular immune responses were monitored after vaccination. Ninety-six percent (23/24) of the patients vaccinated with MAGE-3 protein in AS02B adjuvant elicited a significant anti-MAGE-3 IgG antibody response after 4 vaccinations, and all developed anti-ProtD IgG antibodies. For the detection of T-cell activity, total peripheral blood mononuclear cells were restimulated in vitro with MAGE-3- or ProtD-loaded autologous mature dendritic cells. In 30% of the evaluable patients vaccinated with the adjuvanted recombinant protein, IFNγ production was increased in response to MAGE-3, and 2 patients (14% of evaluable patients) had a concomitant increase in IL-5 production. In 37% and 43% of the patients, respectively, IFNγ or IL-5 production was increased in response to ProtD. It is concluded that vaccination of advanced cancer patients with MAGE-3 self-antigen in AS02B adjuvant is able to elicit MAGE-3-specific antibody and a T-cell response.

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Preliminary results of this study were presented at the Annual Meeting of FOCIS, San Francisco 2002 and the Society for Biological Therapy (SBT), La Jolla, 2002.

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Key Words: cancer immunotherapy; tumor antigen; humoral and cellular response

(*J Immunother* 2004;27:124–135)

Over the last few decades, the field of cancer immunotherapy has been evolving constantly, searching for more efficient treatment, long-lasting clinical benefits, and approaches with fewer side effects. One specific area, active immunotherapy, has seen important changes driven by the discovery of tumor antigens recognized by T cells.[1] Vaccines containing whole tumor cell lysates support the concept of cancer immunotherapy.[2–4] Other approaches are based on vaccination with a single tumor antigen to induce antigen-specific immune responses.

Tumor antigens encoded by the family of MAGE genes are of particular interest in cancer immunotherapy because they are expressed in a variety of malignancies. These include melanoma or other cancers, non-small-cell lung carcinoma (NSCLC), head and neck squamous cell carcinoma, bladder transitional cell carcinoma, and esophagus carcinoma; moreover, MAGE tumor antigens are not detectable in normal tissues except for testis and placenta. Among the 12 members of the MAGE family, MAGE-3 is most frequently expressed in the above-mentioned carcinomas.[5–9]

A number of MHC class I and class II restricted T-cell epitopes have been described for the MAGE-3 tumor antigen.[10] Vaccination with some of these MAGE-3-derived peptides induces immune responses[11–15] and has led to clinical benefits.[12–14,16] Vaccination with peptides is, however, restricted to patients with specific HLA haplotypes capable of interacting with those peptides. Using a whole protein as an immunogen makes it possible to circumvent this restriction and to obtain a broader repertoire of T-cell immune responses.

In cancer, inducing $CD8^+$ and/or $CD4^+$ T cells[17,18] is known to be critical for tumor rejection, and the induction of such a cellular response is promoted by combining the immunogen with an adjuvant. The AS02B adjuvant, which contains

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3-deacylated monophosphoryl lipid A and the QS21 saponin in an oil-in-water emulsion, has been shown to induce a T-helper (Th)1-type cellular response in humans.[19] Furthermore, tumor rejection mediated by both $CD4^+$- and $CD8^+$-specific T cells was observed on vaccination with AS02B-adjuvanted tumor antigens in an animal model.[20] This was the basis for selecting AS02B in our cancer clinical trials. In addition to the T-cell response, the AS02B adjuvant can also induce a strong antibody response.[19,21] However, the potential role of anti-MAGE antibodies in tumor immunotherapy is not clear, and the generation of antibodies to MAGE-3 in this study is mainly monitored as an indication of the immunogenicity of the MAGE-3 self-antigen in man.

To provide additional T-cell help to mount the MAGE-3 immune response, a partial sequence of a nonself antigen was fused to the MAGE-3 antigen.[22,23] Protein D (ProtD), a membrane protein from *Haemophilus influenzae*, was selected because it is known to be immunogenic in humans.[24] Because humans are frequently exposed to this bacterium, primed ProtD-specific T-helper cells are expected to be present at a significant frequency before the vaccination.

In this study, metastatic patients bearing a MAGE-3-positive tumor were vaccinated with different doses of ProtD–MAGE-3 fusion protein (ie, 30, 100, or 300 μg) combined with the AS02B adjuvant. The study was designed to examine safety, immune response, and clinical benefits of the vaccination. Here, we focus on the cellular and antibody response to MAGE-3 and ProtD in vaccinated patients; the safety and clinical responses have been reported elsewhere.[25]

MATERIALS AND METHODS

Trial Eligibility Criteria

All patients were required to have stage III or IV malignancies with measurable lesions of one of the following histologic types: cutaneous melanoma, NSCLC, head and neck squamous cell carcinoma, esophageal or bladder cancer.[25,26] Furthermore, the patients were required to have MAGE-3-positive tumors, which was determined by reverse transcriptase-polymerase chain reaction (RT-PCR) on mRNA extracted from a frozen tumor biopsy,[27] and to express at least 1 of the 3 class I HLA alleles, ie, HLA-A1, HLA-A2, or HLA-B44. In addition, all standard eligibility criteria for phase I/II clinical trials had to be fulfilled.

Vaccine and Immunizations

The MAGE-3 vaccine comprises a purified recombinant fusion protein made up of a partial sequence of the ProtD antigen from *Haemophilus influenzae* fused to the MAGE-3 sequence, and a proprietary GSK adjuvant (GlaxoSmithKline Biologicals, Rixensart, Belgium). For the expression of the recombinant ProtD–MAGE-3 protein, a plasmid was designed encoding for the 18-amino acid periplasmic signal sequence and the first 109 residues of the processed ProtD, 2 unrelated amino acids (methionine and aspartic acid), the amino acid residues 3 to 314 of the MAGE-3 protein, and 2 glycine residues functioning as a hinge region for the subsequent 7 histidine residues. The MAGE-3 cDNA was provided by the Ludwig Institute for Cancer Research, Brussels, Belgium. The construct was engineered into a vector derived from pBR322, which utilizes signals from λ phage DNA to drive the transcription and translation of inserted foreign genes. This vector was used to transform *E. coli* strain AR58 according to standard procedures.[28] Bacteria were grown in LB medium containing 50 μg/mL of kanamycin at 30°C. After heat induction, the ProtD–MAGE-3 protein became detectable as a major band at around 60 kDa when assayed by Western blotting (12.5% SDS-PAGE, reducing conditions, revealed by a rabbit polyclonal antiserum to MAGE-3). After processing of the signal sequence, the recombinant ProtD–MAGE-3 protein comprised 432 amino acid residues. Before purification, the intra- and interprotein disulfide bonds of the protein were chemically reduced, and the thiol groups were blocked with iodoacetamide (carboxyamidation of the protein). The protein was purified from the pellet fraction (30 minutes at 10,000 × *g*) of the cell extract after solubilization with guanidine hydrochloride by using standard chromatography techniques in the presence of urea. The C-terminal His-tag of the protein was required for IMAC (immobilized-metal-chelate affinity chromatography), the key step of the purification. Urea was removed during the last size-exclusion chromatography, and the purified protein was sterilized by filtration through an 0.2-μm membrane. The resulting ProtD–MAGE-3 protein was >90% pure according to SDS-PAGE and Coomassie blue staining analysis.

The GSK adjuvant AS02B (old denomination SBAS2) contained 100 μg of GMP-grade 3-deacylated monophosphoryl lipid A (MPL®, Corixa, Seattle, WA, USA), 100 μg of QS21 (Antigenics, MA, USA), and 250 μL of an oil-in-water emulsion in a final volume of 500 μL per vaccine dose. Three groups of 12 patients each received antigen doses corresponding to 30 (group II), 100 (group III), or 300 μg (group IV) of ProtD–MAGE-3, respectively. A control group (group I) of 3 patients was immunized with the highest dose of antigen (ie, 300 μg) without adjuvant. The vaccination schedule comprised 4 vaccinations at 3-week intervals. Patients whose tumors stabilized or regressed after 4 vaccinations received 2 additional vaccinations at 6-week intervals. The vaccine was administered by intramuscular (IM) route in the upper arm or thigh. For the immunogenicity studies, samples from 28 patients were available for serologic analysis and 21 samples for cellular analysis.

Antigens Used for Immune Readouts

Three recombinant MAGE-3 proteins were engineered in different hosts: *E. coli*, insect cells, and CHO cells. For the expression of the recombinant $MAGE\text{-}3^{E.coli}$ protein in *E. coli*,

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the construct contained the following sequences: 1 methionine residue followed by 7 histidine residues, 3 unrelated amino acids (2 glycine followed by 1 methionine), and the amino acid residues 1 to 314 of the MAGE-3 protein. The construct was engineered into a bacterial vector similar to that used for the expression of the ProtD–MAGE-3 recombinant protein and transformed into the *E. coli* AR58 strain as described above. The recombinant MAGE-$3^{E.coli}$ protein comprised 325 amino acid residues and revealed a band corresponding to a molecular mass of around 46 kDa on 12.5% SDS-PAGE under reducing conditions. The purification of the MAGE-$3^{E.coli}$ protein was carried out as described above for the ProtD–MAGE-3 antigen and resulted in a similar purity (>90%).

The recombinant MAGE-3^{baculo} protein was produced in insect cells with the baculovirus expression system. MAGE-3 cDNA coding for the amino acid residues 1 to 314 of the MAGE-3 protein followed by 6 histidine residues, was engineered into the baculovirus transfer vector pAcYM1. The recombinant baculovirus was isolated and used to infect Hi-5 (Trichophusia Ni) insect cells at a MOI of 0.01 to 0.75. The expressed recombinant MAGE-3^{baculo} protein comprised 320 amino acid residues and ran at around 45–47 kDa on 12% SDS-PAGE gel under reducing conditions. For the purification of the MAGE-3^{baculo} protein, a single IMAC step was used. Although the protein of interest was soluble in the initial material (frozen cell extract), the purification required the presence of urea. Before purification, the protein underwent reduction and carboxyamidation as before but under milder conditions. Urea was removed by dialysis before sterile filtration.

For the expression of the recombinant MAGE-3^{CHO} protein in CHO cells, the MAGE-3 cDNA was subcloned into the vector pEE14 and transfected into CHOK1 cells by calcium phosphate precipitation. A stable clone DGB1#3 expressing MAGE-3 was selected based on the glutamine synthetase–selectable gene marker. Protein expression was induced by decreasing the temperature from 37°C to 33°C, adding sodium butyrate (Sigma, St Louis, MO, USA), and culturing for 96 hours. The construct coded for amino acids 1 to 314 of the MAGE-3 protein, and the recombinant MAGE-3 protein migrated as a 45-kDa band in 10% SDS-PAGE under reducing conditions. Although there was no His-tag in the construct, the purification of the MAGE-3^{CHO} protein relied on IMAC and started from the soluble fraction of the cell extract. Additional ion-exchange chromatography and hydroxyapatite chromatography were used as polishing steps. In this case, no reduction/carboxyamidation of the protein was performed; no urea was used, but 1 mM DTT (dithiothreitol) was added to the elution buffer and was also present in the purified material to prevent oxidation. Upon purification, the MAGE-3^{baculo} protein and MAGE-3^{CHO} protein were >80% pure according to SDS-PAGE and Coomassie blue staining. The MAGE-3 protein does not contain any potential N-glycosylation sites as analyzed with the Protean program of the LaserGene package (DNAStar, Madison, WI, USA).

The tetanus toxoid (TT) antigen was from Chiron Behring (Marburg, Germany), the purified recombinant ProtD antigen, expressed in *E. coli*, was from GSK (Rixensart, Belgium), and the Ovalbumin (Ova) antigen used as a negative control was from Calbiochem (Darmstadt, Germany). All antigens were stored frozen at −80°C, except for TT, which was kept at 4°C.

Preparation of Sera and Peripheral Blood Mononuclear Cells (PBMCs)

The blood sampling for serum and buffy coats or leukapheresis was performed prevaccination, 35 days after 4 vaccinations, and, for patients who received 2 additional vaccinations, 8 days after 6 vaccinations. Human sera and PBMCs from normal healthy donors (blood bank) were used as negative controls or to determine the cutoff in the assays. All sera were stored at −20°C. PBMCs were isolated by Lymphoprep™ (Nycomed Pharma, Oslo, Norway) density gradient centrifugation followed by extensive washing in ice-cold PBS (Gibco, Invitrogen™ Life Technologies, CA, USA). The cells were frozen in 45% heat-inactivated human AB serum (PAA, Linz, Austria), 45% Iscove Modified Dulbecco medium, and 10% DMSO (Sigma) and stored in liquid nitrogen. Viability of the PBMCs after thawing was >80%, as measured by dye exclusion with propidium iodide.

Western Blot

A sample of 2×10^6 MAGE-3–transfected CHO cells were lysed with 665 µL of sample buffer (Tris 25 mM, pH 8.3, 1.4% SDS, 3.6% β-mercaptoethanol, 7.2% glycerol). Cell extract corresponding to 4×10^5 cells was separated on precast 12% SDS-PAGE (Novex, Frankfurt, Germany) along with molecular weight markers from Biolabs (Beverly, MA, USA). The gel was run at 80 V for 15 minutes and then at 200 V for 1 hour. The proteins were transferred onto nitrocellulose in a Mini Trans-Blot cell (Bio-Rad, Hercules, CA, USA) overnight at 30 V and 4°C. The nitrocellulose membrane was then saturated with 10% (w/v) dry milk (Gloria) in PT buffer (PBS, 0.1% Tween 20) for 1 hour at room temperature and cut into 8-mm-wide strips. The strips were incubated in PT buffer for 1 hour at room temperature with patients' sera or mouse anti–MAGE-3 monoclonal antibody mAb54 (1/500) or rabbit anti–MAGE-3 polyclonal serum LRM69 (1/1000). The strips were washed 3 times with PT buffer and incubated for another 30 minutes at room temperature with an anti–human IgG–alkaline phosphatase (AP) conjugate or an anti–mouse or anti–rabbit IgG–AP conjugate (all from Promega, Madison, WI, USA). After 3 washes with PT buffer, the strips were developed with NBT/PICB (nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-1-phosphate, Promega). The reaction was stopped in water, and the nitrocellulose sheets were scanned (Nokia).

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MAGE-3 and ProtD ELISA

The MAGE-3^{baculo} antigen was coated overnight at 4°C on 96-well plates (Maxisorb Immunoplate, Nunc, Roskilde, Denmark) at a final concentration of 1 μg/mL in PBS without Ca^{2+} or Mg^{2+} (Cambrex, East Rutherford, NJ, USA). ELISA plates were then saturated with 5% (w/v, final concentration) dry milk (Gloria) in PBS for 1 hour at 37°C. Serial dilutions (2-fold) of patients' sera, starting at 1/20 dilution in PTB buffer (PBS, 0.05% Tween 20, 1% BSA) + PVA (polyvinyl alcohol) 0.1% (vol/vol) were incubated overnight at 4°C. Plasma from MAGE-3–vaccinated patients, whose sera were shown to be positive in a Western blot (as described above), were included on each plate and served as standard and positive control. ELISA plates were washed 3 times with PT buffer and incubated for 2 hours at 22°C with a secondary antibody (sheep anti–human IgG–peroxidase conjugate, Amersham, Buckinghamshire, England) diluted 1/5000 in PTB buffer + PVA 0.1%. The plates were washed again as before and incubated for 15 minutes with TMB (tetramethylbenzidine, BioRad) resuspended in citrate 0.1 M, acetic acid 0.1 M, pH 5.8. The reaction was stopped with 0.4 N H_2SO_4, and the plates were analyzed at 450 nm (reference 620 nm) using an ELISA reader (Molecular Devices E-max). All volumes were 100 μL/well except for the saturation step, where 200 μL was used. The titers of the sera pre- and postvaccination were determined by comparison with the standard plasma of known titer value and are expressed as ELISA Units per milliliter (EU/mL). The cutoff value was 27 EU/mL and corresponded to 3 standard deviations (SD) above the mean titer of a healthy control population (n = 60). The signals detected in the MAGE-3^{baculo} ELISA, using the standard and positive control, were abrogated with purified MAGE-3^{CHO} antigen but not with Ova, showing that the references used in the assay were specific for the native MAGE-3 antigen. In addition, no anti-His tail-specific antibody response was found in sera of patients exhibiting a high anti-MAGE-3^{baculo} antibody response (data not shown).

ProtD ELISA was carried out as described for MAGE-3 except that PVA was omitted in all buffers. The cutoff was 100 EU/mL for antibody response. A patient was considered a MAGE-3 or ProtD vaccine responder when the postvaccination titer was above the respective cutoff value and at least twice the patient's own baseline value.

For statistical evaluation, a one-way ANOVA was performed with the $\log_{10}$-transformed anti–MAGE-3 and anti-ProtD antibody titers. Differences between titers were regarded as statistically significant at P values below 0.05.

Preparation of Dendritic Cells

Media and additives were from Gibco if not otherwise stated. All cultures were performed at 37°C and 5% CO_2. Between 100 and 150 × 10^6 pre- and postvaccination PBMCs were thawed and pooled for the preparation of dendritic cells (DCs) as previously described.[29] The DC populations were phenotyped using a panel of mAbs (CD1a, CD80, CD83, CD3, CD4, and CD14; Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed on a FACScan® with the Cell Quest® software (Becton Dickinson) as described.[30]

Preparation of Protein-Pulsed DCs

DCs collected after a 6-day culture were loaded overnight with 20 μg/mL of MAGE-3^{baculo}, MAGE-3$^{E.\ coli}$, ProtD, TT, or Ova as described previously.[31] The protein-loaded DCs were then irradiated at 6000 Rad and used either fresh (first stimulation) or frozen (second stimulation and specificity assay). The same batch of protein-loaded mature autologous DCs was used for the stimulation of PBMCs pre- and postvaccination.

In Vitro Sensitization of PBMCs with Protein-Pulsed DCs

The in vitro sensitization of PBMCs was performed according to Murakami.[32] Briefly, nonsorted PBMCs (0.1 × 10^6 cells/well) were cocultured with protein-loaded mature autologous DCs (E/T = 10) in 200 μL of RPMI 1640 supplemented with 10% heat-inactivated human AB serum (PAA) and GPS (L-glutamine 2 mM, penicillin 100 IU/mL, streptomycin 100 μg/mL) (96-well plates, round bottom). Three antigenic stimulations per sample were run in parallel: MAGE-3$^{E.\ coli}$, ProtD, and TT. An average of 24 wells was prepared for each antigenic stimulation. The pre- and postvaccination PBMCs were stimulated in parallel to avoid interday variability. After a 3-day culture, IL-2 (20 IU/mL, Boehringer Ingelheim, Germany) was added, and after a 1-week culture, the effector cells were restimulated with protein-loaded mature DCs (0.01 × 10^6 per well). IL-2 (20 IU/mL) was added the day of the restimulation and whenever the culture was split up.

Assessment of T-Cell Reactivity Using Cytokine Release Assay

The stimulated effector cells were tested for specificity 7 days after the second stimulation as described by Steller et al.[33] For each antigenic stimulation, the stimulated cells were harvested, pooled, and cocultured for 24 hours with protein-loaded mature autologous DCs (0.1 × 10^6 cells/well; E/T = 1). The effector cells from each antigenic stimulation (MAGE-3$^{E.\ coli}$, ProtD, and TT) were tested against the respective relevant antigen (MAGE-3^{baculo}, ProtD, and TT) and against Ova as an irrelevant protein. Culture of effector cells alone and protein-loaded DCs alone were used as negative controls. All cultures were set up in duplicates in 200 μL of RPMI 1640 supplemented with 10% of heat-inactivated human AB serum (PAA) and GPS (96-well plates, round bottom). After 24 hours, the supernatants from each coculture were collected and tested by ELISA for IFNγ and IL-5 content (IFNγ and IL-5 Quantikine enzyme-linked immunosorbent assay kits, Biosource, Camarillo, CA, USA). The detection threshold was 100 pg/mL for

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both cytokines. The geometric mean of the cytokine productions was calculated for all cultures.

Definition of Specific and Positive Cytokine Production

Specific cytokine productions were calculated as the amount of cytokine produced in 24 hours (pg/mL) by effector PBMCs in the presence of the relevant antigen (MAGE-3^{baculo}, ProtD, or TT) minus the production induced by the irrelevant antigen (Ova).

The release of IFNγ or IL-5 by 0.1×10^6 PBMCs in culture was regarded positive when the specific 24-hour cytokine production was above the cutoff and the signal/noise ratio was at least 5. The cutoff values for IFNγ and IL-5 correspond to 3 SD above the mean specific cytokine production determined in cultured PBMCs from 12 healthy donors. The cutoff for the IFNγ release was 1500 pg/mL for MAGE-3- or ProtD-, and 500 pg/mL for TT-stimulated cells. Similarly, the cutoff for the IL-5 release was 500 pg/mL for MAGE-3 or ProtD and 100 pg/mL for TT. The IFNγ release by prevaccination PBMCs of patient 7057 was classified as positive although the signal/background ratio was only 3.

Criteria for Evaluabiliy of Patients and Vaccine Responders

Patients were considered evaluable if their TT response (IFNγ and IL-5 release by PBMCs after stimulation with TT) was positive and stable between pre- and postvaccination with a 2-fold variation tolerated. The evaluable patients were considered as MAGE-3 or ProtD vaccine responders when the positive cytokine production of their postvaccination PBMCs was at least 3 times the amount of cytokines produced by their prevaccination PBMCs.

RESULTS

The present study reports the immunologic responses in advanced metastatic patients bearing a MAGE-3–positive tumor, mostly melanoma, after vaccination with three doses (30, 100, or 300 μg) of a ProtD–MAGE-3 fusion protein adjuvanted with AS02B. Fifty-seven patients were enrolled in the study, 39 of whom received at least 4 vaccinations. Because pre- and postimmunization sera and PBMCs could not be collected from all patients for medical reasons, antibody responses were analyzed for 28 patients, and CMI responses were analyzed for 21 patients. The clinical responses to vaccination of the 57 patients have already been described.[25] The objective of this second part of the clinical trial was to evaluate if vaccination with a self antigen was able to elicit antibody and/or T-cell responses in tumor patients.

Antibody Response

To examine whether the vaccine had induced a MAGE-3–specific antibody response, pre- and postvaccination sera of several vaccinated patients were analyzed by Western blot (Fig. 1). Because the vaccine contained a recombinant MAGE-3 protein produced in *E. coli*, the Western blot was carried out with a MAGE-3 protein produced in a different expression system (MAGE-3^{CHO}) to allow for a MAGE-3–specific response. On the strips incubated with post- rather than prevaccination sera, a signal corresponding to the MAGE-3 antigen was observed, indicating the presence of anti–MAGE-3 antibodies in the postvaccination sera. These antibodies were specific for native MAGE-3 protein because they recognized a MAGE-3^{CHO} antigen that had not undergone reduction/carboxyamidation and did not contain a His-tail.

To quantify the antibodies, 2 specific IgG ELISAs were developed in which plates were coated with MAGE-3^{baculo} protein or with the whole ProtD (~300 amino acids). Twenty-eight sera were examined before and after 4 vaccinations, of which 6 were also examined after 6 vaccinations (see Table 1). In the prevaccination samples, about 40% of the patients had a

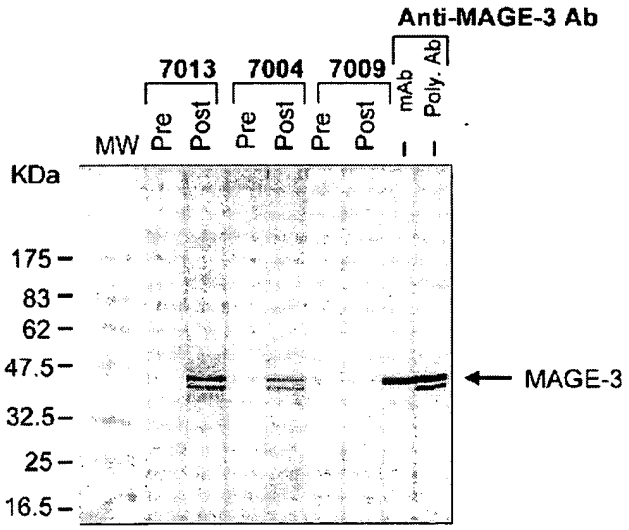

FIGURE 1. Western blot using a lysate of MAGE-3–transfected CHO cells immunostained with the sera of vaccinated patients. Lysates of MAGE-3–transfected CHO cells were subjected to 12% SDS-PAGE under reducing conditions and blotted onto a nitrocellulose membrane. Molecular weight (MW) standards are indicated on the left side of the gel. The MAGE-3^{CHO} protein has a molecular weight of 39 kDa and was immunodetected with a murine anti–MAGE-3 monoclonal antibody (mAb54, 1/500) or a rabbit anti–MAGE-3 polyclonal antibody (LRM69, 1/1000). The Western blot revealed 2 bands of the MAGE-3 protein, of which the lower band is likely to be a degradation product of the MAGE-3 protein. Individual strips were incubated with pre- and postvaccination sera of 3 patients vaccinated with 30 μg of adjuvanted recombinant MAGE-3 protein [7013 (1/500), 7004 (1/500), and 7009 (1/100); group II] and immunostained with a phosphatase-labeled anti-human IgG.

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TABLE 1.

Vaccine	Patients	Anti–MAGE-3 Ab Titer (EU/mL)			Anti-ProtD Ab Titer (EU/mL)		
		Pre	Post-4	Post-6	Pre	Post-4	Post-6
Group I	7002	10	10	—	178	100	—
Control group	7020	8	206	—	172	254	—
300 μg ProtD-MAGE-3 without AS02B	7023	8	16	—	100	100	—
GMT		9	32	na	145	136	na
Group II	7004	12	946	—	133	24,532	—
30 μg ProtD–MAGE-3 with AS02B	7009	21	76	—	100	943	—
	7013	8	517	—	100	2,153	—
	7036	10	1,465	—	100	3,498	—
	7037	12	11,742	19,370	244	320	637
	7040	11	285	—	100	376	—
	7046	10	88	311	276	7,487	2,864
	7049	9	733	—	406	3,421	—
	7051	10	23,921	14,876	100	7,481	8,898
GMT		11	869	na	149	2,514	na
95% CI		9–14	192–3,922	na	98–228	830–7,614	na
Group III	7015	9	—	973	100	—	1,741
100 μg ProtD–MAGE-3 with AS02B	7018	11	938	—	100	3,922	—
	7019	13	1,467	—	100	7,339	—
	7022	nt	479	—	1849	18,621	—
	7024	9	1,033	—	100	782	—
	7025	9	752	841	100	792	491
	7050	9	1,517	—	100	448	—
GMT		10	959	na	152	2,032	na
95% CI		8–11	666–1,385	na	55–521	631–7,759	na
Group IV	7031	9	847	—	147	2,743	—
300 μg ProtD-MAGE-3 with AS02B	7033	9	367	—	134	2,595	—
	7038	11	11	—	112	3,472	—
	7042	10	1,524	—	100	1,692	—
	7044	10	3,696	—	100	4,358	—
	7048	10	678	—	100	3,624	—
	7054	15	2,104	—	135	926	—
	7055	8	294	—	119	561	—
	7057	8	157	550	100	365	623
GMT		10	477	na	115	1,710	na
95% CI		8–11	126–1,802	na	102–130	863–3,391	na
GMT* (Groups II–IV)		10	711	1,852	138	2,118	1,393
95% CI*		9–11	364–1,390	291–11,784	104–183	1,268–3,574	429–4,530

positive ProtD titer. Most of the preexisting anti-ProtD antibody titers were low (<250 EU/mL) except for patient 7022, who showed a titer of 1849 EU/mL. This overall ProtD serology was expected because ProtD is derived from a bacterium often encountered by humans. Although spontaneous anti–MAGE-3 antibody production has been reported in about 10% of melanoma patients,[34] in the present study none of the 28 patients who received 4 vaccinations exhibited a MAGE-3 response before vaccination.

In the groups that received 4 vaccinations with the antigen combined with the AS02B adjuvant, all but one of 24 patients developed a MAGE-3–specific antibody response. As

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expected, all patients developed a ProtD-specific antibody response. In the nonadjuvanted group, only 1 out of 3 patients developed a MAGE-3 antibody response, and none of them developed a ProtD response.

The magnitude of the antibody response was also examined after 4 vaccinations (see Table 1 and Fig. 1A). For both the MAGE-3 and the ProtD response, the mean antibody titers of patients receiving the adjuvanted protein were more than 10 times higher than the mean titer of patients receiving the nonadjuvanted protein (group 1), regardless of the vaccine dose. Neither anti–MAGE-3 nor anti-ProtD antibodies were statistically different among the 3 adjuvanted groups (one-way ANOVA, Table 1 and Fig. 1A).

To study the kinetics of the antibody response (see Fig. 2B), all antibody titers from adjuvanted groups obtained for a given time point were averaged regardless of the vaccine dose and followed over time. A statistically significant difference was observed between pre- and postvaccination antibody responses to both MAGE-3 and ProtD, but no difference was seen after 4 or 6 vaccinations, suggesting that the immune response persisted at a plateau level after the fourth immunization.

No correlation was found between the anti–MAGE-3 and the anti-ProtD antibody titers at individual levels, which could be ascribed to the heterogeneity of the preexisting antibody response to ProtD. The anti–MAGE-3 IgG subclasses were defined by ELISA (data not shown). Out of 19 sera tested after 4 vaccinations, the predominant IgGs (>70%) found in the sera were IgG1 (16 patients), IgG2 (2 patients), and IgG3 (1 patient). Patients whose anti–MAGE-3 and anti-ProtD titers were available were sorted according to the stage of disease: melanoma stage III, melanoma Stage IV M1a, melanoma Stage IV M1b, and nonmelanoma. For each group, the GMT and the 95%CI were calculated for both the anti–MAGE-3 and the anti-ProtD serology. The statistical analysis (one-way ANOVA, $P < 0.05$) revealed that neither anti–MAGE-3 nor anti-ProtD antibodies were statistically different among the 4 groups.

In conclusion, vaccination with ProtD–MAGE-3 in AS02B adjuvant gave rise to statistically significant anti–MAGE-3 and anti-ProtD IgG responses. The dose of antigen did not affect the magnitude of the antibody response, whereas the presence of the AS02B adjuvant had a strong impact.

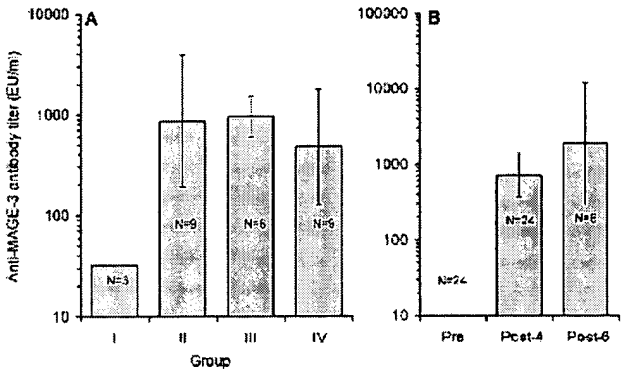

FIGURE 2. Anti–MAGE-3 antibody response in vaccinated patients. Anti–MAGE-3 IgG antibody titers were quantified by an ELISA assay using a purified MAGE-3^{baculo} protein. Geometric mean titers (GMT) and 95% confidence intervals (95% CI) of the antibody titers were calculated for different populations. **(A)** Effect of adjuvant and dose of antigen: antibody titers after 4 vaccinations of patients vaccinated with antigen alone (group I, 300 μg) or vaccinated with adjuvanted antigen equivalent to 30 μg (group II), 100 μg (group III), and 300 μg (group IV) of recombinant protein. Group 1 consisted of only 3 patients; therefore, no 95% CI was calculated. **(B)** Kinetic: antibody titers pre- and postvaccination (post-4 and post-6) of patients vaccinated with adjuvanted protein.

Assay for Monitoring the Cellular Response

To detect the MAGE-3 cellular response, first an ex vivo ELISPOT assay[35] as well as a 5-day proliferation assay were performed on PBMCs. The cytokine production of T-helper cell subsets was also studied.[36] IFNγ ELISPOT assays implying stimulation of PBMCs with the MAGE-3 protein turned out to be nonspecific. Lymphoproliferation and analysis of cytokine production by $CD4^+$ T cells also failed to provide conclusive results. These negative results prompted us to perform an in vitro restimulation assay using protein-pulsed autologous dendritic cells to stimulate the effector cells. A cytokine (IFNγ and IL-5)-release assay was then used to measure the activity of the stimulated T cells. To detect a MAGE-3–specific response, MAGE-3 proteins from 2 different expression systems were used for the stimulation and for the cytokine-release assay, respectively. Both recombinant MAGE-3 proteins contained a His-tag for purification, which was located at the N-terminus of the MAGE-3$^{E.coli}$ antigen and at the C-terminus of the MAGE-3^{baculo} antigen. The amino acid sequence surrounding the His-tail was different in the 2 constructs to avoid recording of a possible anti-His T-cell response.

Determination of Evaluable Patients Based on the Cytokine Production in Response to TT

In the cancer population enrolled in this trial, a number of parameters can impact on the immune status of the patient, eg, the nature of treatment undergone before vaccination and change in tumor burden. Therefore, the use of a positive and stable internal control is essential for conclusions to be drawn from the MAGE-3 responses. The TT response was used as an internal control because most of the human adults exhibit a T-cell response directed toward this antigen.[37,38] Moreover, in the in vitro assay, the TT antigen undergoes the same process in terms of uptake and processing as the MAGE-3 and ProtD antigens and hence is a relevant control for the experiment. Evaluable patients should, therefore, comply with 2 criteria (at

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the 2 time points considered): (1) a specific production of cytokine by PBMCs in response to stimulation with TT and, (2) because the TT response is expected not to be affected by the MAGE-3 vaccination, the cytokine production has to remain stable as an indicator that a MAGE-3 response is not caused by global immune stimulation. Pre- and postvaccination PBMCs from 21 patients were examined in the in vitro restimulation assay (Fig. 3). Five of the 21 patients were nonevaluable for IFNγ, and 7 patients nonevaluable for IL-5 secretion because their TT-induced cytokine production at 1 or both of the 2 time points examined was negative. Hence, 16 patients were analyzed for IFNγ and 14 patients for IL-5 production in response to MAGE-3 and ProtD (Fig. 3D).

Spontaneous Cytokine Production in Response to MAGE-3 and ProtD

As described for other tumor antigens such as NY-ESO-1[39,40] or Her2/*neu*,[41] a spontaneous MAGE-3-specific T-cell response can be observed in cancer patients. In the present study, production of IFNγ before vaccination was detected in 3 patients (7037, 7024, and 7057), and of IL-5 in 1 patient (7037) (Fig. 3A). None of these patients exhibited an anti–MAGE-3 IgG antibody response in their prevaccination sera, but, interestingly, 2 of those patients (7037 and 7057) showed a clinical benefit after vaccination in terms of tumor regression or stabilization. As expected, prevaccination IFNγ production in response to ProtD was also observed (in patients 7036, 7046, 7022, 7024, 7039, and 7057).

IFNγ Production in Response to Vaccination

With regard to the IFNγ production on MAGE-3 stimulation, 5 out of 16 evaluable patients were considered as vaccine responders (Fig. 3A,D). The range of IFNγ produced varied from 1500 to 45,000 pg/mL; the increase above the patient's prevaccination baseline was variable, ranging from 3-fold to 77-fold (see Fig. 3A). Among the MAGE-3 vaccine responders, 1 patient (7037) had a prevaccination MAGE-3–specific response, and the other 4 patients were negative. One patient showed a stable response to MAGE-3 before and after vaccination (7057). Six out of 16 evaluable patients were ProtD vaccine responders (Fig. 3B,D), of whom only 2 patients (7046 and 7019) had a preexisting IFNγ response to ProtD. IFNγ production ranged from 1500 to 54,000 pg/mL, and the increase above the patient's baseline varied from a 3-fold to a 40-fold increase (see Fig. 3B). Five patients showed a stable ProtD response between pre- and postvaccination, which was as high as 6000 pg/mL or above, indicating that the cytokine production might have reached a plateau level. Four of 5 MAGE-3 vaccine responders were also ProtD vaccine responders, and 1 showed a high and stable ProtD-induced IFNγ production before and after vaccination. All MAGE-3 vaccine responders were also ProtD responders, but not vice versa (7029 and 7015).

IL-5 Production in Response to Vaccination

Two out of 14 evaluable patients were MAGE-3 vaccine responders in terms of IL-5 production showing a 6- and 12-fold increase above baseline (Fig. 3A,D). Neither of the 2 patients exhibited a preexisting response to MAGE-3. One patient (7057) showed a MAGE-3 response postvaccination but no increase compared with the prevaccination baseline. Six out of 14 evaluable patients were ProtD vaccine responders (6- to 30-fold increase, Fig. 3B,D), of whom only 1 patient (7036) had a prevaccination ProtD IL-5 response. Two out of 14 patients showed a stable ProtD response before and after vaccination. In terms of IL-5 production, MAGE-3 vaccine responders were also ProtD vaccine responders but not vice versa. In 2 patients, the secretion of both cytokines was stimulated by MAGE-3, and in 5 patients this dual effect was induced by ProtD. Two patients had a stable production of both IL-5 and IFNγ between pre- and postvaccination.

In summary, a MAGE-3–specific T-cell response as shown by increased IFNγ production on MAGE-3 stimulation could be detected in 30% (5/16) of the evaluable patients vaccinated with the recombinant ProtD–MAGE-3 antigen formulated in AS02B adjuvant. Fourteen percent of the patients experienced a concomitant increase in IL-5 production. A ProtD-specific increase in IFNγ and IL-5 production was observed in 37% (6/16) and 43% (6/14) of the evaluable patients, respectively. Eighty-six percent of the MAGE-3 vaccine responders also developed a ProtD vaccine response, irrespective of the type of cytokine released. Hence, the MAGE-3 responses observed were likely induced by the vaccination.

DISCUSSION

In a phase I/II study, patients with metastatic MAGE-3–positive solid tumors, mainly melanoma, were vaccinated with MAGE-3 protein formulated with or without adjuvant AS02B and evaluated for toxicity, immune response, and clinical response. The MAGE-3 vaccine was well tolerated and was associated with clinical benefit in 6/57 enrolled patients and 6/39 patients who received at least 4 vaccinations.[25] The results reported here show that a specific immune response to MAGE-3 could be induced in advanced cancer patients using MAGE-3 protein combined with AS02B adjuvant. Significant antibody titers were induced in all but 1 patient, even with antigen dose as low as 30 μg, whereas specific T-cell responses to MAGE-3 were detectable after vaccination in roughly 30% of evaluable patients.

Use of AS02B Adjuvant

An adjuvant system made of MPL and QS21 in an oil-in-water emulsion very similar to that used in this trial has previously been applied to vaccines for infectious diseases and shown to significantly promote T_H1 and antibody responses to malaria[21] and hepatitis B (Pierre Vandepapeliere, *personal*

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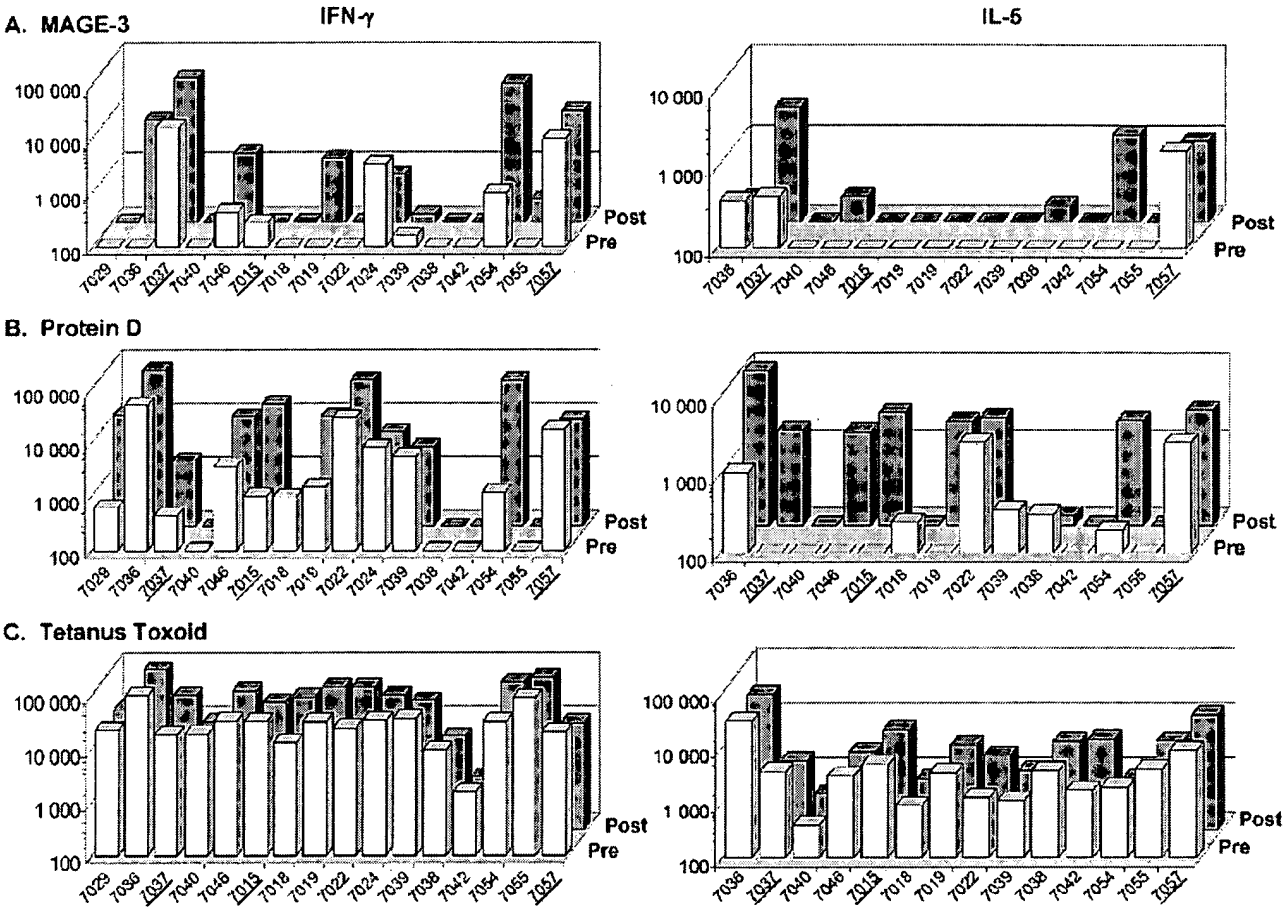

D. Summary

		Group I		Group II							Group III							Group IV				
Patients		7020	7023	7009	7028	7029	7036	7037	7040	7046	7015	7018	7019	7021	7022	7024	7039	7038	7042	7054	7055	7057
MAGE-3	IFN-γ					-	+	+	-	+	-	-	+		-	?	-	-	-	+	-	=
	IL-5						-	+	-	-	-	-	-		-		-	-	-	+	-	=
ProtD	IFN-γ					+	=	+	-	+	+	-	+		=	=	=	-	-	+	-	=
	IL-5						+	+	-	+	+	-	+		=		-	-	-	+	-	=
TT	IFN-γ	ne	ne	ne	ne	e	e	e	e	e	e	e	e	ne	e	e	e	e	e	e	e	e
	IL-5	ne	ne	ne	ne	ne	e	e	e	e	e	e	e	ne	e	ne	e	e	e	e	e	e

FIGURE 3. Cytokine production on in vitro restimulation of patients' PBMCs with MAGE-3–, ProtD-, or TT protein–loaded autologous DC cells. Total PBMCs of evaluable patients taken before and after 4 vaccinations (for patient 7046 post-6) were cocultured and restimulated (after 1 week) with irradiated autologous mature DCs loaded with MAGE-3$^{E.\ coli}$ **(A)**, ProtD **(B)**, or TT **(C)**. The effector cells were tested for specificity against the relevant protein: MAGE-3^{baculo} **(A)**, ProtD **(B)**, or TT **(C)** or against ovalbumin. IFNγ and IL-5 released into the supernatant were quantified by ELISA. The specific cytokine productions were calculated from the amount of cytokine produced in the presence of the relevant antigen (MAGE-3^{baculo}, ProtD, or TT) minus that induced by the irrelevant antigen (Ova) and are expressed in picograms per milliliter. Open bars represent prevaccination PBMCs; filled bars postvaccination PBMCs. The underlined identification numbers indicate clinical responders. **(D)** Summary of the immune status of all patients analyzed based on cytokine production: (+), vaccine responders; (=), no increase in cytokine production between pre- and postvaccination; (−), no specific cytokine production; (?), unclear; (e) evaluable or (ne) nonevaluable according to the TT criteria. Nonevaluable samples are marked (gray).

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communication) immunogens. Unlike the viral or parasitic antigens, tumor antigens such as MAGE-3 are regarded as weak antigens in terms of immunogenicity. The MAGE-3 antigen is expressed during embryogenesis and is silenced in adult tissues. Although a true immune tolerance toward MAGE-3 has not been documented, one might argue that high-avidity T cells could have been deleted and that only low-frequency of MAGE-3–specific T cells remained. Therefore, raising an immune response toward a self antigen was not taken for granted. To our knowledge, this is the first observation that vaccination with the MAGE-3 protein combined with a potent immunostimulatory adjuvant resulted in a strong impact on antibody titer and the induction of detectable T-cell responses with a predominance of IFNγ production over IL-5 production.

Antibody Response

The protein D fusion protein was incorporated into the immunogen as a source of exogenous T-helper cell epitopes with the objective of inducing immune responses that increase local cytokine level, thus also promoting induction of immune responses to self antigens contained in the ProtD–MAGE-3 immunogen.[22,23] It is difficult to conclude from the results of our trial that the ProtD moiety played a role in promoting induction of an immune response to MAGE-3 because a control vaccine without ProtD was not used, and most patients responded to both ProtD and MAGE-3 by producing specific IgG, irrespective of the antigen dose but dependent on the presence of the AS02B adjuvant in the vaccine. However, we can conclude that the presence of ProtD in the vaccine had no deleterious effect because the relative antibody response between ProtD and MAGE-3 did not change significantly with increased number of vaccinations, indicating that carrier-induced epitopic suppression[42,43] did not occur.

To analyze the T_H1/T_H2 profile of the response induced by the MAGE-3 vaccine, the relative amounts of the different IgG subclasses have been determined (data not shown). Although the correlation of IgG subclasses with the T_H1/T_H2 profile is less obvious in humans than in mice,[44] we showed a predominance of IgG1 and IgG3 subclasses, suggesting that a T_H1-type immune response has been induced by the vaccine favoring an antitumor response.[45] The role of MAGE-3–specific antibodies in tumor immunotherapy is not established, but it is not excluded that MAGE-3 antibodies could contribute to tumor rejection by binding to shed tumor antigens and promoting local inflammatory processes.

Cellular Response

In the present study, an improved method to monitor cellular response has been employed, taking advantage of several important features. (1) Two in vitro stimulations in the presence of protein-loaded DCs enhanced the sensitivity of the assay. (2) The antigens used to monitor the cellular response were designed to ensure the specificity of the MAGE-3 responses. The antigens used for the stimulation and in the cytokine-release assay were produced in 2 different expression systems to avoid nonspecific responses as a result of contaminant proteins. (3) The immune response to TT, which was not supposed to be affected by a MAGE-3 vaccine, had to remain stable between pre- and postvaccination. This criterion was used as an indicator of stability of the global immune response. (4) A whole protein was used for restimulation to detect reactivity to all potential MAGE-3 epitopes. Both IFNγ and IL-5 were quantified because they are indicative of the T_H1/T_H2 immune response profile.[46] Our results suggest that both T_H1 and T_H2 components of the immune response are implicated. Although the frequency of the MAGE-3 T-cell response was not quantified, the amount of IFNγ produced in response to MAGE-3 could be substantial, which might reflect a large expansion of MAGE-3 T cells. This is an important observation because a high T-cell frequency has been shown to be associated with tumor clearance in mice.[47]

The in vitro restimulation assay allowed us to identify and classify vaccine responders, of whom 31% and 37% produced IFNγ in response to stimulation with MAGE-3 and ProtD, respectively, whereas 14% and 43% produced IL-5 after the restimulation with MAGE-3 and ProtD, respectively. The number of vaccine responders may seem to be low when compared with other approaches.[13] However, qualitative and quantitative biases are introduced by the in vitro restimulation assay; eg, only precursor T cells able to proliferate in vitro are detected. The protein picked up by DCs is directed to the endocytic compartment; therefore, mostly $CD4^+$ T cells are stimulated. As a result, a number of other T cells, such as immediate effector cells or $CD8^+$ T cells, potentially raised on vaccination are not recorded with that assay. For most patients, only 1 postvaccination time point was examined, at 5 weeks after the fourth immunization. Because it has been reported that tumor antigens are able to induce only transient responses,[14,48] it is possible that the MAGE-3 cellular response was missed by the assay. Finally, it has been described for tumor-bearing patients that T cells traffic to the tumor and therefore disappear from the peripheral blood.[49]

CONCLUSIONS AND PERSPECTIVES

In the cancer immunotherapy field, a correlation between the immune responses induced by vaccination and the clinical responses has been difficult to establish. Although some correlation has been reported by some groups,[50,51] there is no consensus in the scientific community of the correlates of protection or the appropriate immune readout for monitoring cancer vaccine clinical trials. In the present study, clinical responses were observed in 6 out of 12 patients with metastatic nonvisceral lesions,[25] but no clear correlation was found between those clinical responses and the cytokine profile, the anti–MAGE-3 antibody titers, or IgG subclass. In our view, a correlation between the clinical and the immune response is

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more likely to be observed in less-advanced cancer patients[52] and by investigating a larger number of patients. This approach would make it possible to optimize the vaccine and identify relevant immune parameters to be used as surrogate markers for the disease. However, the in vitro restimulation has validated the induction of MAGE-3–specific T cells in patients after vaccination with an adjuvanted MAGE-3 protein. This prompts us to further explore the MAGE-3 T-cell response and, by using ex-vivo assays[53] in less advanced patient populations, to examine the frequency of MAGE-3–specific T cells, the type of cytokine they produced, and the phenotype of those T cells.

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A MAGE-3 Peptide Presented by HLA-DR1 to CD4$^+$ T Cells That Were Isolated from a Melanoma Patient Vaccinated with a MAGE-3 Protein[1]

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"Cancer-germline" genes such as those of the *MAGE* family are expressed in many tumors and in male germline cells, but are silent in normal tissues. They encode shared tumor-specific Ags, which have been used in therapeutic vaccination trials of cancer patients. *MAGE-3* is expressed in 74% of metastatic melanoma and in 50% of carcinomas of esophagus, head and neck, bladder, and lung. We report here the identification of a new MAGE-3 peptide, which is recognized by three different CD4$^+$ T cell clones isolated from a melanoma patient vaccinated with a MAGE-3 protein. These clones, which express different TCRs, recognize on HLA-DR1 peptide ACYEFLWGPRALVETS, which corresponds to the MAGE-3$_{267-282}$ and the MAGE-12$_{267-282}$ protein sequences. One of the T cell clones, which expresses LFA-1 at a high level, lysed tumor cells expressing DR1 and MAGE-3. Another of these DR1-restricted CD4$^+$ clones recognized not only the MAGE-3/12 peptide but also homologous peptides encoded by genes *MAGE-1, 2, 4, 6, 10,* and *11*. *The Journal of Immunology,* 2003, 171: 219–225.

Human tumors bear Ags that are recognized by autologous T lymphocytes and that are highly specific for tumors. Some of these Ags are shared by many tumors of various histological types (1). Shared tumor-specific Ags, such as those encoded by gene *MAGE-3*,[4] are attractive candidates for therapeutic vaccination aimed at eliciting antitumoral T cell responses in cancer patients (2). First, the strict tumoral specificity of these Ags ought to ensure the absence of damage on normal tissues after immunization. Second, the presence of these Ags on many tumors makes it possible to apply the same vaccine to a large set of patients. Therefore, these Ags have been used for small-scale therapeutic vaccination trials of melanoma patients with detectable disease. The vaccines consisted of an antigenic peptide, a protein, a pox family recombinant virus carrying a MAGE-3 sequence, and dendritic cells (DCs) pulsed with an antigenic peptide (3–8). No significant toxicity was observed. Complete or partial clinical responses have been observed in only a small proportion of the patients, but the rate of tumor regressions appears to be well above the rates of spontaneous regressions that have been reported (9).

The use of defined antigenic peptides greatly facilitates the immunological monitoring because the presumed target of the anti-vaccine T cells is completely defined. It allows, for instance, the use of HLA-peptide tetramers, which are very sensitive tools for the detection of T cell responses in patients vaccinated with tumor-specific peptide (10, 11). Alternatively, a protein-based vaccine offers the advantage that antigenic peptides binding to a broad set of HLA molecules can be processed from the vaccine, so that the patients do not have to be selected according to their HLA. In a recently completed trial, patients with a measurable tumor expressing *MAGE-3* were vaccinated with escalating doses of a recombinant MAGE-3 protein combined for most of the patients with adjuvant SBAS-2 (8). The immunization schedule included four i.m. injections at 3-wk intervals of a fusion protein containing the MAGE-3 portion, a lipidated protein D derived from *Haemophilus influenzae* at its N terminus, and a sequence of several histidine residues at its C terminus (Prot.D/MAGE-3/His). Among the 33 melanoma patients who were evaluable for tumor response, we observed two partial responses, two mixed responses, and one stabilization. Interestingly, one of the partial responses occurred in a melanoma patient who did not receive the adjuvant. Thus, the Prot.D/MAGE-3/His protein given alone, without adjuvant, could also have the capacity to immunize, which might be due to the properties of its lipidated protein D.

This is presently investigated in a new trial in which the protein is given intradermally and subcutaneously without adjuvant. In this study, a partial response occurred upon vaccination: most of the in-transit and lymph node metastases of patient DDHK2 completely regressed after six injections of the MAGE-3 protein. Three different anti-MAGE-3 CD4$^+$ T cell clones were isolated from the blood cells of this patient. Here we are describing the identification of a new MAGE-3 antigenic peptide that is presented to these three T cell clones by HLA-DR1 molecules.

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[4] Abbreviations used in this paper: DC, dendritic cell; EBV-B cell, EBV-transformed B cell; Ii, invariant chain; LNGFR, low-affinity nerve growth factor receptor; IRES, internal ribosome entry site.

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Materials and Methods

Melanoma patient DDHK2

Patient DDHK2 was included in clinical trial LUD 99–003. It was approved by the Protocol Review Committee of the Ludwig Institute for Cancer Research and by the ethics committee Commission d'Ethique Biomédicale Hospitalo-Facultaire de la Faculté de Médecine de l'Université de Louvain. The informed consent form was signed by the patient. Patient DDHK2 was vaccinated with recombinant protein Prot.D/MAGE-3/His, which was produced in *Escherichia coli* as a fusion protein with lipidated protein D derived from *H. influenzae* at the N terminus and a sequence of several histidine residues at the C terminus of the MAGE-3 protein. Injections were given without adjuvant at 3-wk intervals, intradermally and s.c. Blood cells were collected 2 wk after the fourth injection of the MAGE-3 protein.

Cell lines, media, and reagents

The EBV-transformed B (EBV-B) cell lines and the tumor cell lines were cultured in IMDM (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS (Life Technologies), 0.24 mM L-asparagine, 0.55 mM L-arginine, 1.5 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Human rIL-2 was purchased from Eurocetus (Amsterdam, The Netherlands), IL-7 from Genzyme (Cambridge, MA), GM-CSF from Schering Plough (Brinny, Ireland), and TNF-α from R&D Systems (Abingdon, U.K.). Human recombinant IL-4, IL-6, and IL-12 were produced in our laboratory. Anti-HLA-DR Ab L243 was obtained from the American Type Culture Collection (Manassas, VA). Anti-HLA-DP Ab B7/21 and anti-HLA-DQ Ab SPV-L3 were kindly provided by A. Mulder (Department of Immunohematology, University of Leiden, Leiden, The Netherlands). The Abs were used at a 1/5 dilution of the culture supernatant.

MAGE-3 proteins

Two different MAGE-3 proteins were used. One was produced in our laboratory in *Spodoptera frugiperda* (Sf9) insect cells using a baculovirus expression system (BD PharMingen, San Diego, CA), as described previously (12). It will be referred to hereafter as protein MAGE-3^{insect}. The other MAGE-3 protein, hereafter referred to as protein MAGE-$3^{bacteria}$, was produced in *E. coli* by GlaxoSmithKline Biologicals (Rixensart, Belgium), as previously reported (13). It contains a sequence of several histidine residues at the N terminus of the protein. The recombinant MAGE-3 protein used in the vaccine is a fusion protein with a lipidated protein D derived from *H. influenzae* at its N terminus and a sequence of several histidine residues at the C terminus of the protein (Prot.D/MAGE-3/His). The inclusion of the first 109 residues of the protein D as a fusion partner was expected to improve the immunogenicity and to provide the vaccine protein with additional bystander help properties, whereas the inclusion of a His affinity tail facilitated the purification of the fusion protein. The protein was produced in *E. coli* and extensively purified to eliminate bacterial contaminants.

Construction of the retrovirus encoding MAGE-3, MAGE-1, MAGE-4, and invariant chain (Ii)-MAGE-3

The retroviral vectors encoding MAGE-1, 3, and 4 were derived from the LXSN backbone, an expression vector derived from Moloney murine leukemia virus (Clontech, Palo Alto, CA). Respectively, they encode the full-length MAGE-1, 3, and 4 under the control of the long terminal repeat and the truncated form of the human low-affinity nerve growth factor receptor (LNGFR) driven by the SV40 promoter. For transduction, EBV-B cell lines were cocultivated with irradiated Am12 vector-producing cells in the presence of polybrene (0.8 mg/ml) for 72 h. A pure population of transduced cells was obtained by immunoselection with anti-LNGFR mAb 20.4 (American Type Culture Collection) and goat anti-mouse IgG FITC (BD Biosciences, San Jose, CA). For producing the retrovirus encoding Ii-MAGE-3, the sequence encoding a truncated form of LNGFR was amplified from plasmid pUC19-ΔLNGFR, which was kindly provided by C. Traversari (Istituto Scientifico H.S. Raffaele, Milano, Italy). Briefly, LNGFR was ligated into pCR2.1 to an internal ribosome entry site (IRES) sequence, derived from the encephalomyocarditis virus. The IRES-ΔLNGFR sequence was then transferred into pMFG-Ii80, which encodes the first 80 aa of the human invariant chain (Ii80). A complete MAGE-3 cDNA was then ligated downstream Ii80 into pMFG-Ii80-IRES-ΔLNGFR, allowing the simultaneous expression of the Ii-MAGE-3 fusion protein and the truncated LNGFR receptor. The procedure for transducing cell lines has been described previously (14).

DCs and $CD4^+$ responder T cells

DCs were obtained by culturing monocytes in the presence of IL-4 (200 U/ml) and GM-CSF (70 ng/ml) in RPMI 1640 medium supplemented with asparagine-arginine-glutamine and 1% autologous plasma. One-fourth of the medium was replaced by fresh medium and cytokines every 2 days. On day 7, the nonadherent cell population was used as a source of enriched DCs, as described previously (15). Rosetted T cells were treated with NH_4Cl (160 mM) to lyse the sheep erythrocytes, and they were washed. $CD4^+$ T lymphocytes were isolated from rosetted T cells by positive selection using magnetic microbeads coupled to an anti-CD4 mAb (Miltenyi Biotech, Bergisch Gladbach, Germany) and by sorting through a MACS, as recommended by the manufacturer (Miltenyi Biotech).

Mixed lymphocyte/DC culture

DCs (5×10^5/ml) were incubated at 37°C, 5% CO_2, for 20 h in complete RPMI 1640 medium supplemented with IL-4, GM-CSF, and TNF-α (1 ng/ml) in the presence of MAGE-$3^{bacteria}$ (20 μg/ml). The small amount of TNF does not induce the maturation of DCs, as measured by CD83 expression. However, we cannot exclude that additional TNF produced by T cells or activation through CD40-CD40 ligand during the coculture led to DC maturation. Cells were washed and added at 10^4 per round-bottom microwell to 10^5 autologous $CD4^+$ T lymphocytes in 200 μl of IMDM supplemented with asparagine-arginine-glutamine and 1% autologous plasma in the presence of IL-6 (1000 U/ml) and IL-12 (10 ng/ml). The $CD4^+$ T lymphocytes were restimulated on days 7, 14, 21, and 28 with autologous DCs freshly loaded with MAGE-$3^{bacteria}$ and were grown in IMDM supplemented with asparagine-arginine-glutamine and 1% autologous plasma (hereafter referred to as complete IMDM) supplemented with IL-2 (10 U/ml) and IL-7 (5 ng/ml). Aliquots of each microculture (~5,000 cells) were stimulated on day 42 with ~20,000 autologous EBV-B cells loaded for 20 h with 20 μg/ml MAGE-$3^{bacteria}$, MAGE-3^{insect}, or OVA. After 20 h of coculture in round-bottom microwells and in 100 μl of complete IMDM supplemented with IL-2 (25 U/ml), IFN-γ released in the supernatant was measured by ELISA using reagents from Medgenix Diagnostics-Biosource (Fleurus, Belgium).

$CD4^+$ T cell clones

Cells from positive microcultures were cloned by limiting dilution, using irradiated autologous EBV-B cells transduced with retro-Ii.MAGE-3 (5×10^3–2×10^4 cells) as stimulator cells. Irradiated allogeneic LG2-EBV cells (5×10^3–10^4) were used as feeder cells. $CD4^+$ T cell clones were supplemented once a week with fresh culture medium in the presence of IL-2 (50 U/ml), IL-7 (5 ng/ml), and IL-4 (5 U/ml). The cytokines produced by the $CD4^+$ T cell clones upon contact with the Ag were measured after overnight coculture using the Cytometric Bead Array kit from BD PharMingen.

TCR analysis

For TCR analysis, 3×10^5 cells from each clone were used for extracting RNA with the Tripure reagent (Boehringer Mannheim, Mannheim, Germany) and were converted to cDNA at 42°C for 90 min with 200 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies). TCR Vα and Vβ usage was assessed by PCR amplification by using a complete panel of Vα- or Vβ-specific sense primers and Cα and Cβ antisense primers, respectively (16). Primers were chosen on the basis of described panels of TCR V region oligonucleotides and with alignments of TCR sequences available at the International Immunogenetics Database (http://imgt.cines.fr). Each PCR product was purified and sequenced to obtain a complete identification of the CDR3 region.

Recognition assays with peptides

Peptides were synthesized on solid phase using 9-fluorenylmethoxycarbonyl chemistry for transient NH_2-terminal protection and were characterized using mass spectrometry. All peptides were >90% pure, as indicated by analytical HPLC. Lyophilized peptides were dissolved at 5 mg/ml in 10 mM acetic acid and 10% DMSO and were stored at −20°C. EBV-B cells were distributed at 20,000 cells per round-bottom microwell and were incubated for 2 h at 37°C in the presence of the different peptides, the indicated concentrations representing their concentrations during the incubation step. $CD4^+$ T lymphocytes (5000) were added in 100 μl of complete IMDM (Life Technologies) supplemented with IL-2 (25 U/ml). Supernatants were harvested after 20 h of coculture, and IFN-γ production was measured by ELISA. The peptides used in Fig. 4*A* correspond to the MAGE-$1_{260-275}$, MAGE-$2_{267-282}$, MAGE-$3_{267-282}$, MAGE-$4_{268-283}$, MAGE-$6_{267-282}$, MAGE-$10_{292-307}$, MAGE-$11_{270-285}$, and MAGE-$12_{267-282}$ protein sequences.

Recognition assays with cell lysates

EBV-B cells (5×10^4) and tumor cells (5×10^4) were lysed in 50 μl of complete RPMI 1640 by three cycles of rapid freeze-thawing. HLA-DR1 monocyte-derived DCs (2.5×10^4) were then added to the lysates in 150 μl of complete RPMI 1640 supplemented with IL-4 (100 U/ml) and GM-CSF (70 ng/ml) and were kept at 37°C for 24 h. DCs were washed and 5000 $CD4^+$ lymphocytes were added in 150 μl of complete IMDM supplemented with IL-2 (25 U/ml). Supernatants were harvested after 20 h of coculture, and IFN-γ production was measured by ELISA. Experiments with lysates of tumor cells were performed with autologous DCs, and the experiments with lysates of EBV-B cells were performed with DR1-matched DCs.

Recognition of tumor cells

Tumor cells were distributed at 20,000 cells per round-bottom microwell together with 5,000 $CD4^+$ T lymphocytes in 100 μl of complete IMDM in the presence of IL-2 (25 U/ml). Supernatants were harvested after 20 h of coculture, and IFN-γ production was measured by ELISA. For measuring lytic activity, cells were labeled with 100 μCi of $Na^{51}CrO_4$, and 1000 targets were added to the T cells at different E:T ratios. Chromium release was measured after 4 h of incubation at 37°C.

HLA-DR peptide binding assay

Purification of HLA-DR molecules and peptide binding assays was performed as previously described (17, 18). Briefly, HLA-DR molecules were purified from EBV-B homozygous cell lines by affinity chromatography. They were incubated with different concentrations of competitor peptide and an appropriate biotinylated peptide. The biotinylated peptides were the following: HA 306–318 (PKYVKQNTLKLAT) for DRB1*0101 (1 nM, pH 6), DRB1*0401 (30 nM, pH 6), DRB1*1101 (20 nM, pH 5), and DRB5*0101 (5 nM, pH 5.5); YKL (AAYAAAKAAALAA) for DRB1*0701 (10 nM, pH 5); A3 152–166 (EAEQLRAYLDGTGVE) for DRB1*1501 (10 nM, pH 4.5); MT 2–16 (AK TIAYDEEARRGLE) for DRB1*0301 (100 nM, pH 4.5); B1 21–36 (TERVR LVTRHIYNREE) for DRB1*1301 (200 nM, pH 4.5); and LOL 191–210 (ES WGAVWRIDTPDKLTGPFT) for DRB3*0101 (5 nM, pH 5.5). The binding was evaluated in a fluorescence assay. Data were expressed as the peptide concentration that prevented binding of 50% of the labeled peptide (IC_{50}). Averages were obtained from at least two independent experiments. Unlabeled forms of the biotinylated peptides were used as reference peptides to assess the validity of each experiment.

Results

Derivation of anti-MAGE-3 $CD4^+$ T cell clones

Monocyte-derived DCs of patient DDHK2 were loaded overnight with a MAGE-3 protein produced in bacteria ($MAGE\text{-}3^{bacteria}$). In two independent experiments, a total of 192 microcultures of 10^5 $CD4^+$ T cells and 10^4 stimulator DCs were set up. To favor the activation of Th1 lymphocytes, the culture medium was supplemented with IL-12. After four weekly restimulations with protein-loaded DCs, the responder cells were tested for their ability to secrete IFN-γ upon stimulation with the Ag. Considering that a large proportion of the $CD4^+$ T cells obtained in our initial experiments appeared to be directed against bacterial contaminants, we used a protein produced in insect cells ($MAGE\text{-}3^{insect}$) for this test. Seven microcultures that specifically produced IFN-γ were cloned and restimulated with autologous EBV-B cells transduced with a retroviral construct encoding a truncated human invariant chain (Ii) fused with the MAGE-3 protein (retro-Ii.MAGE-3) (13, 19). In this chimeric protein, signals within the Ii should target the MAGE-3 protein to the class II Ag-processing compartments (19).

Anti-MAGE-3 $CD4^+$ T cell clones were obtained from only three of the seven microcultures that were cloned. These clones recognized autologous EBV-B cells either loaded with 20 μg/ml of protein $MAGE\text{-}3^{bacteria}$ or $MAGE\text{-}3^{insect}$ or transduced with retro-Ii.MAGE-3 (Fig. 1*A*). Each of these three clones was able to lyse EBV-B cells expressing the Ii-MAGE-3 fusion protein (Fig. 1*B*). They produced high amounts of IFN-γ and TNF upon contact with the Ag and smaller amounts of IL-2, IL-4, and IL-10 (Fig. 1*C*). Each of these three clones had a different TCR (Table I).

Identification of the antigenic peptides

We tested for recognition by each of the three clones autologous EBV-B cells pulsed with a set of peptides of 16 aa, overlapping by 12 residues and covering the complete MAGE-3 sequence. Two overlapping peptides, GSDPACYEFLWGPRAL ($MAGE\text{-}3_{263\text{–}278}$) and

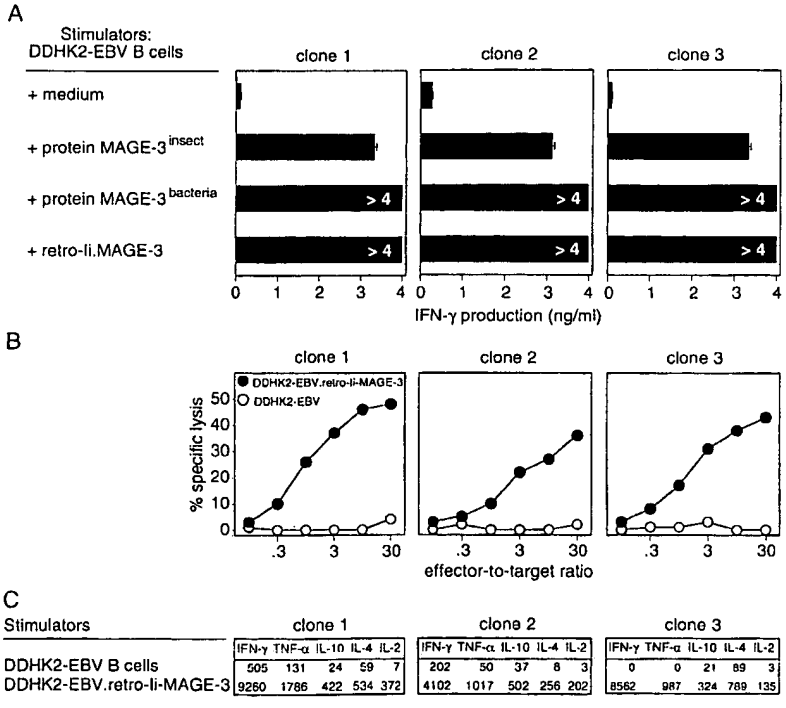

FIGURE 1. Recognition of a MAGE-3-derived Ag by three $CD4^+$ T cell clones. *A*, Stimulator cells were the autologous EBV-B cells either loaded with 20 μg/ml protein $MAGE\text{-}3^{bacteria}$ or $MAGE\text{-}3^{insect}$ or transduced with a retroviral construct encoding a truncated human Ii fused with the MAGE-3 protein (retro-Ii.MAGE-3). Stimulator cells (20,000) were cocultured overnight with 5,000 $CD4^+$ T cells. The concentration of IFN-γ produced in the medium was measured by ELISA. The results shown represent an average of triplicate cocultures. *B*, HLA-DR1 DDHK2-EBV B cells are autologous to the $CD4^+$ T cells. DDHK2-EBV.retro-Ii.MAGE-3 were obtained by transduction of DDHK2-EBV with a retroviral construct encoding a truncated human Ii fused with the MAGE-3 protein (retro-Ii.MAGE-3). Targets were chromium labeled for 1 h and incubated for 4 h with the CTL at indicated E:T ratios. *C*, Stimulator cells (20,000) were cocultured overnight with 5,000 $CD4^+$ T cells in a total volume of 150 μl. The concentration of cytokines produced in the medium was measured by cytometric bead array analysis. Numbers represent pg/ml.

Table I. *TCR sequences of the three anti-MAGE-3 CD4⁺ T cell clones*[a]

Clone	TCRα	V	CDR3	J
α chain				
Clone 1	V9-2*01-J49*01	TCAGCGGTGTACTTC	TGTGCTCTTGAGAACACCGGTAACCAGTTCTATTTTGGG	ACAGGGACAAGTTTGACGGTCATT
		S A V Y F	C A L E N T G N Q F Y F G	T G T S L T V I
Clone 2	V36/DV7*04-J37*01	TCGGCCATCTACCTC	TGTGCTGTGGGTGTCTGGCAACACAGGCAAACTAATCTTTGGG	CAAGGGACAACTTTACAAGTAAAA
		S A I Y L	C A V V S G N T G K L I F G	Q G T T L Q G I
Clone 3	V27*04-J52*01	ACAGGCCTCTACCTC	TGTGCAGGAAGGGGAAGAGGTACTAGCTATGGAAAGCTGACATTTGGA	CAAGGGACCATCTTGACTGTCCAT
		T G L Y L	C A G R G G R G T S Y G K L T F G	Q G T I L T V H
β chain				
	TCRβ	V	CDR3	J
Clone 1	V28*01-J2-2*01	ACATCTATGTACCTC	TGTGCCAGCAGACCCTTCCCGGGGAGCTGTTTTTGGA	GAAGGCTCTAGGCTGACCGTACTG
		T S M Y L	C A S R F F P G E L F F G	E G S R L T V L
Clone 2	V9*01-J2-1*01	TCAGCTTTGTATTTC	TGTGCCAGCAGCGTGTACTCCAATGAGCAGTTCTTCGGG	CCAGGGACACGGCTCACCGTGCTA
		S A L Y F	C A S S V Y S N E Q F F G	F G T R L T V L
Clone 3	V6-3*01-J1-2*01	ACATCTGTGTACTTC	TGTGCCAGCAGTCTGACAGGGACCAACTATGGCTACACCTTCGGT	TCGGGGACCAGGTTAACCGTTGTA
		T S V Y F	C A S S L T G T N Y G Y T F G	S G T R L T V G

[a] V and J rearrangements were attributed according to the nomenclature available at http://imgt.cines.fr.

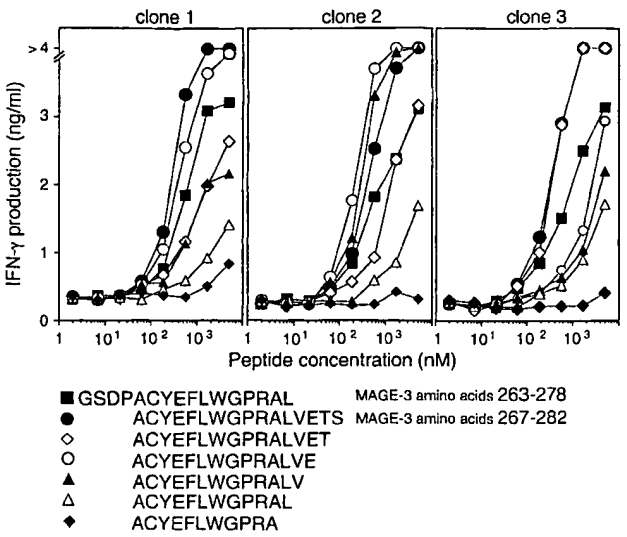

FIGURE 2. Recognition of peptide ACYEFLWGPRALVETS by each of the three CD4⁺ clones. DDHK2 EBV-B cells were distributed in microwells (2×10^4 cells) and incubated for 2 h with the indicated concentrations of peptides. A total of 5×10^3 cells from each autologous CD4⁺ T cell clone was added, and the presence of IFN-γ in the supernatant was measured by ELISA after overnight coculture. The results shown represent an average of triplicate cocultures.

ACYEFLWGPRALVETS (MAGE-$3_{267-282}$), stimulated the production of IFN-γ by all three clones (Fig. 2). The latter peptide is also encoded by MAGE-12 (MAGE-$12_{267-282}$). Testing shorter peptides indicated that the most efficiently recognized was slightly different for each clone: ACYEFLWGPRALVETS for clone 1, ACYEFLWG PRALVE for clone 2, and both ACYEFLWGPRALVET and ACY EFLWGPRALVETS for clone 3 (Fig. 2).

The antigenic peptide is presented to T cells by HLA-DR1 molecules

For each of the three clones, the recognition of autologous EBV-B cells loaded with peptide MAGE-$3_{267-282}$ was abolished by an anti-HLA-DR Ab, but not by Abs against HLA-DP or HLA-DQ (data not shown). Patient DDHK2 was typed HLA-DR1, DR15, and DR51. Peptide ACYEFLWGPRALVETS was loaded on several EBV-B cell lines sharing HLA-DR molecules with patient

Table II. *MAGE-3 peptide ACYEFLWGPRALVETS is presented by HLA-DR1*[a]

EBV-B Cell Line	Serological Specificity	IFN-γ Production (pg/ml) Clone 1	Clone 2	Clone 3
DR1 positive				
DDHK2	DR1 DR15 DR51	>4000	>4000	>4000
LB1158	DR1 DR13 DR52	2439	2029	1698
LB831	DR1 DR7 DR53	2037	1418	1371
LB2138	DR1 DR13	1810	2046	2053
DR1 negative				
LB650	DR7 DR15 DR51 DR53	83	160	150
LB1870	DR15 DR53 DR7	88	104	119
LB1856	DR15	49	187	164
LB2095	DR13 DR15 DR51 DR52	22	172	125

[a] EBV-B cells were incubated for 2 h with 5 μg/ml peptide ACYEFLWGPRALVETS, washed, and incubated (20,000 cells) with 5,000 cells of clone 1, 2, or 3. IFN-γ production was measured by ELISA after overnight coculture. The results shown represent the average of triplicate cocultures.

Table III. *Binding of peptide MAGE-3$_{267-282}$ to multiple HLA-DR molecules*[a]

HLA-DR Alleles	IC_{50} (nM) of Reference Peptides[b]	IC_{50} (nM) of MAGE-3$_{267-282}$	Ratios (IC_{50} MAGE-3/ IC_{50} Reference)
DR1	2 (±1)	0.4 (±0.2)	0.2
DR3	350 (±0)	>10,000	
DR4	40 (±4)	730 (±4)	18
DR7	10 (±3)	1,200 (±350)	120
DR11	25 (±0)	150 (±0)	6
DR13	390 (±140)	>10,000	
DR15	15 (±0)	220 (±35)	15
DRB3	20 (±0)	>10,000	
DRB4	40 (±19)	2,800 (±1,400)	70
DRB5	15 (±6)	170 (±140)	11

[a] The capacity of the peptide MAGE-3$_{267-282}$ to bind multiple HLA-DR molecules was investigated on purified DR molecules in competition assays using fluorescent reference peptides (±SD). To facilitate the comparison, data are also presented as the ratio between the IC_{50} of the MAGE peptide and that of the reference peptide. A ratio <10 indicates peptides with a high affinity for a given HLA class II molecule, whereas a ratio >10 corresponds to intermediate binders.

[b] Reference peptides are described in *Materials and Methods*.

DDHK2. All and only those expressing DR1 were recognized by the three clones when loaded with the peptide (Table II).

The binding of peptide MAGE-3$_{267-289}$ to purified HLA-DR molecules of various allotypes was tested in competition assays using HLA-DR-specific biotinylated peptides (Table III). The MAGE-3 peptide displayed a very high affinity for DR1, binding more efficiently than the reference peptide (20). It also bound to the following molecules, listed by decreasing affinity: DR11, DRB5, DR15, DR4, DRB4, and DR7. No binding was observed on DR3, DR13, and DRB3.

Recognition of tumor cells

Proteins that carry an endosomal targeting sequence are directed to the endosomes, enabling the cell to present on class II molecule peptides derived from internal proteins. For instance, some melanocytic proteins are specifically targeted to melanosomes, which are organelles derived from the endocytic compartment (21). MAGE proteins do not contain signal sequences or endosomal targeting sequences. Therefore, class II-restricted MAGE peptides are not expected to be presented by the tumors expressing *MAGE*.

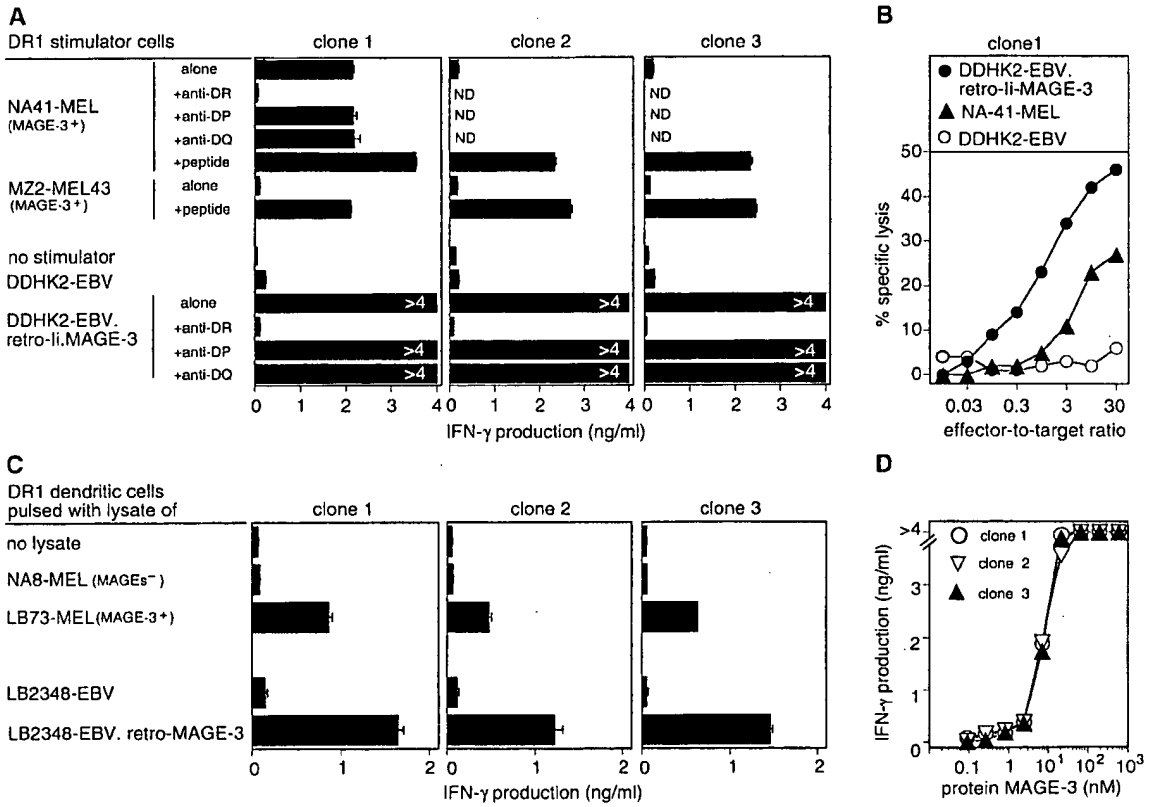

FIGURE 3. Recognition of tumor cell lines and of DCs loaded with tumor-derived MAGE-3. HLA-DR1 melanoma lines NA41-MEL and MZ2-MEL.43 express *MAGE-3*. HLA-DR1 DDHK2-EBV B cells are autologous to the $CD4^+$ T cells. DDHK2-EBV.retro-li.MAGE-3 were obtained by transduction of DDHK2-EBV with a retroviral construct encoding a truncated human Ii fused with the MAGE-3 protein (retro-li.MAGE-3). *A*, Direct recognition of tumor cell lines. Cells were distributed in flat-bottom microwells (2×10^4 cells per well) and, if indicated, pulsed for 2 h with 5 μg/ml peptide ACYEFLWGPRALVETS and washed. If indicated, mAbs directed against HLA-DR, DP, or DQ were added in the medium. Five thousand $CD4^+$ T cells were added to the stimulator cells. IFN-γ production was measured by ELISA after 20 h of coculture. The results shown represent an average and SD of triplicate cocultures. ND, No data. *B*, Lysis of a tumor cell line by clone 1. Targets were chromium labeled for 1 h and incubated for 4 h with the CTL at indicated E:T ratios. The results shown represent an average of triplicates. *C*, Recognition of DCs loaded with tumor-derived MAGE-3. The tumor cells and EBV-B cells do not express DR1 and were lysed by freeze-thawing. Melanoma cell line LB73-MEL expresses *MAGE-3*, whereas NA8-MEL does not express *MAGE-3* or other MAGE-A genes. HLA-DR1 DCs (2.5×10^4 cells per well) were cultured for 24 h with lysates at the equivalent of two cells per DC. After washing, the DCs were incubated with 5×10^3 T cells per well. IFN-γ production was measured after 20 h by ELISA. The results shown represent an average of triplicate cocultures. *D*, Titration of the MAGE-3 protein. HLA-DR1 DCs (2×10^4 cells per flat-bottom microwell) were cultured for 24 h with different concentrations of the MAGE-$3^{bacteria}$ protein, washed, and incubated with 5×10^3 cells per well from each $CD4^+$ T cell clone. IFN-γ production was measured by ELISA after 20 h of coculture. The results shown represent an average and SD of triplicate cocultures.

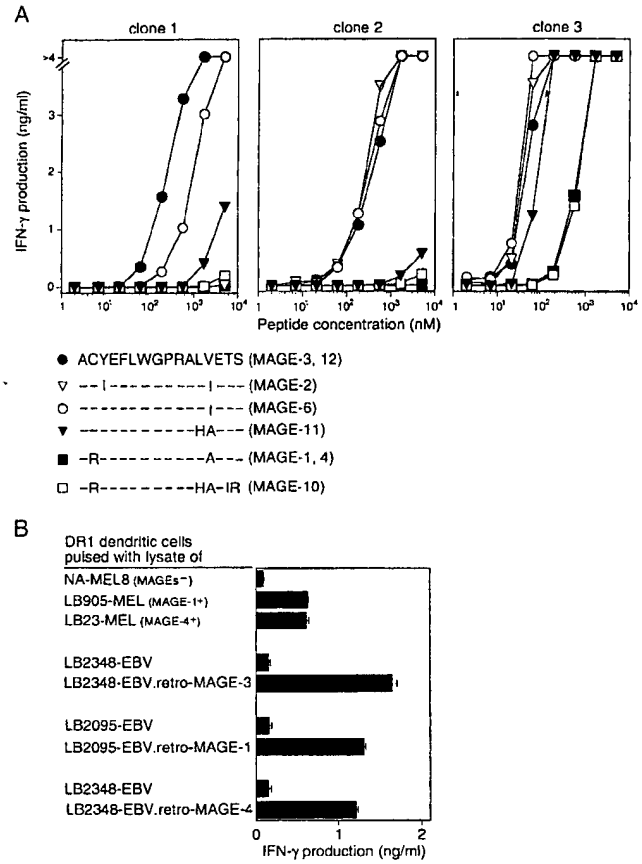

FIGURE 4. Recognition of different MAGE peptides by the three CD4$^+$ T cell clones. *A*, DDHK2-EBV B cells (2×10^4) were incubated for 2 h with the indicated peptides. A total of 5×10^3 cells from each autologous CD4$^+$ T cell clone was added, and IFN-γ production in the supernatant was measured by ELISA after overnight coculture. *B*, Recognition of DCs loaded with tumor-derived MAGE proteins. The tumor cells and EBV-B cells do not express DR1 and were lysed by freeze-thawing. Melanoma cell line NA8-MEL does not express *MAGE-3* or other MAGE-A genes. LB905-MEL expresses *MAGE-1* but not other MAGE-A genes; LB23-MEL expresses *MAGE-4* but not other MAGE-A genes. HLA-DR1 DCs (2.5×10^4 cells per well) were cultured for 24 h with lysates at the equivalent of two cells per DC. After washing, the DCs were incubated with 5×10^3 T cells per well. IFN-γ production was measured after 20 h by ELISA. The results shown represent an average of triplicate cocultures.

In accordance with this, we described two DR13-restricted MAGE-3 peptides and one DR15-restricted MAGE-1 peptide that were not recognized by CD4$^+$ T cell clones at the surface of tumor cells (13, 22). However, we and others have identified MAGE-3 peptides presented by DR11 and DP4 that were recognized on tumor cells expressing MAGE-3 (12, 23). How these peptides are processed and presented is unclear.

Here, we tested the production of IFN-γ by the three CD4$^+$ clones stimulated by the melanoma lines NA41-MEL and MZ2-MEL.43, which express the *DR1* and *MAGE-3* genes. Clone 1 recognized the NA41-MEL cells, indicating that MAGE-3 antigenic peptide can be naturally processed and presented by melanoma cells (Fig. 3*A*). Recognition of NA41-MEL cells was abolished by an anti-HLA-DR Ab, but not by Abs against HLA-DP or HLA-DQ. This CD4$^+$ clone also lysed NA41-MEL cells and the autologous EBV-B cells that were transduced with retro-Ii. MAGE-3 (Fig. 3*B*). The absence of recognition of MZ2-MEL.43 cannot be explained by a lower level of expression of *MAGE-3*, because expression was equivalent in NA41-MEL and MZ2-MEL.43, as measured by semiquantitative RT-PCR (data not shown). The two other CD4$^+$ clones were not stimulated by the melanoma lines, although the avidity of the three clones was equivalent. We compared the three clones by testing their ability to be stimulated by DCs loaded with decreasing concentrations of protein MAGE-3, and we observed that cells incubated with <10 nM protein stimulated the three clones to produce 2 ng/ml IFN-γ (Fig. 3*D*). We tentatively concluded that the direct recognition of tumors is not only dependent on the adequate expression of MAGE-3 or a high avidity of the CD4$^+$ T cell clone.

Stimulation of CD4$^+$ T cells can also occur via presentation of tumor debris by APCs. Here, we have pulsed DR1 DCs with lysates of DR1^{NEG} cells expressing MAGE-3. Each of the three CD4$^+$ T cell clones released IFN-γ in response to tumor-derived MAGE-3 processed by the DR1 DCs (Fig. 3*C*). They also specifically recognized the DCs pulsed with a lysate of cells transduced with a retrovirus construct containing the coding sequence of MAGE-3.

Recognition of the homologous MAGE peptides

Peptide ACYEFLWGPRALVETS is encoded by *MAGE-3* and *MAGE-12*. Homologous peptides encoded by other *MAGE* genes are only slightly different from the MAGE-3/12 peptide (Fig. 4*A*). All of the peptides share the central part EFLWGPRA. They were tested for their ability to stimulate the CD4 clones. The MAGE-2 and MAGE-6 peptides were recognized by clones 2 and 3 as efficiently as was the MAGE-3 peptide, despite the replacement of a tyrosine or a valine by isoleucines (Fig. 4*A*). The MAGE-11 peptide was recognized by clone 3, despite the replacement of 2 aa. The MAGE-1, MAGE-4, and MAGE-10 peptides were also recognized by clone 3, but only when 20 times higher peptide concentrations were used. DR1 DCs were incubated with lysates of EBV-B cells expressing *MAGE-1*, *3*, or *4* and were tested for their ability to stimulate clone 3 to produce IFN-γ (Fig. 4*B*). As expected, clone 3 was stimulated with cells pulsed with lysates of *MAGE-3*-expressing cells. Importantly, it was also stimulated with cells pulsed with lysates of MAGE-1- or MAGE-4-expressing cells, demonstrating that clone 3 is able to recognize naturally processed MAGE-1 or MAGE-4 proteins and not only synthetic peptides.

Discussion

The new MAGE-3 antigenic peptide described here, ACYEFL WGPRALYETS (MAGE-$3_{267-282}$), was presented to three CD4$^+$ T cell clones by HLA-DR1 molecules, which are expressed in ~18% of Caucasians, ~11% of Africans, and ~6% of Asians. This peptide is also encoded by *MAGE-12*. It can also bind efficiently to HLA-DR11 and to a lesser extent to DRB5, DR15, DR4, DRB4, and DR7 molecules. Interestingly, the homologous peptides encoded by *MAGE-1*, *2*, *4*, *6*, *10*, or *11* are only slightly different from the MAGE-3 peptide. Because these homologous peptides are also recognized by one of the CD4$^+$ T cell clones, we can suppose that such T cells may also be activated in patients injected with the MAGE-3 peptide or a MAGE-3 protein. These T cells will be able to recognize not only the *MAGE-3*-expressing cells, but also other tumor cells that do not express *MAGE-3* or that have lost the expression of *MAGE-3*, provided that they express one of the other *MAGE* genes. The activation of these T cells should reduce the emergence of Ag-loss variants in vaccinated patients.

Stimulation of the CD4$^+$ antitumoral T cells could result from the recognition of tumor Ags on macrophages or DCs that have endocytosed tumor cell debris. This recognition could stimulate the CD4$^+$ T cells to provide "help" that would magnify the CTL

response (24). We have shown here that anti-MAGE-3.DR1 $CD4^+$ T cells can recognize DCs loaded with lysates. Another mode of action of $CD4^+$ T cells could result from a direct recognition of Ags presented by class II molecules on the surface of the tumor cells, resulting in the destruction of these cells. Tumor cells of many types have been shown to bear class II molecules (25, 26). We have also shown here that some anti-MAGE-3.DR1 $CD4^+$ T cells can recognize and lyse NA41-MEL cells, which express *MAGE-3*.

The three $CD4^+$ T cell clones were unable to recognize MZ2-MEL.43 cells, which also express *MAGE-3*. Preliminary experiments have shown that adhesion molecule ICAM-1 was less expressed on MZ2-MEL.43 than on NA41-MEL, which was recognized by clone 1 (data not shown). Moreover, LFA-1, the ligand of ICAM-1, was more highly expressed on clone 1, the only clone that directly recognized NA41-MEL (data not shown). This suggests that the direct recognition of tumor cells is not only dependent on an adequate expression of MAGE-3. A high expression of adhesion molecules seems to also be required, on both the T cells (LFA-1) and the tumor cells (ICAM-1).

The first clinical trial with HLA-class II-restricted MAGE peptides was reported recently. Mature monocyte-derived DCs were loaded with keyhole limpet hemocyanin and MAGE-3.DP4, MAGE-3.DR13, tyrosinase.DR4, or gp100.DR4 peptide. Autologous Ag-loaded DCs were injected in metastatic cancer patients (7). Eight of 16 evaluable patients exhibited stable disease, and one patient experienced a complete regression of metastases in lung and pancreas. IFN-γ-producing cells were detected by ELISPOT analyses in most patients, after in vitro stimulation of the blood cells with the MAGE-3 peptides, but not when cells were stimulated with the tyrosinase or the gp100 peptide.

It is interesting to note here that patient DDHK2 was injected with the entire MAGE-3 protein and that we isolated $CD4^+$ T cells that were specific for only one MAGE-3 peptide, despite the use of DCs loaded with the entire MAGE-3 protein as stimulators. Therefore, this peptide might be a immunodominant peptide in HLA-DR1 patients. This hypothesis could be tested by the analysis of the immune response of several other DR1 patients vaccinated with a MAGE-3 protein.

A possibility for this monitoring is the use of the peptide that can be used either to select and amplify peptide-specific T cells in vitro or to directly label TCRs with soluble HLA class II tetramers presenting the relevant peptide (27). A reliable and quantitative monitoring of the specific $CD4^+$ T cell response will be important for the improvement of vaccination strategies. This is difficult because these responses do not appear to be massive, as suggested by a quantitative monitoring of the $CD8^+$ T cell response directed against a MAGE-3.A1 peptide (11).

Acknowledgments

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Vaccine-Induced CD4$^+$ T Cell Responses to MAGE-3 Protein in Lung Cancer Patients[1]

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MAGE-3 is the most commonly expressed cancer testis Ag and thus represents a prime target for cancer vaccines, despite infrequent natural occurrence of MAGE-3-specific immune responses in vivo. We report in this study the successful induction of Ab, CD8$^+$, and CD4$^+$ T cells in nonsmall cell lung cancer patients vaccinated with MAGE-3 recombinant protein. Two cohorts were analyzed: one receiving MAGE-3 protein alone, and one receiving MAGE-3 protein with adjuvant AS02B. Of nine patients in the first cohort, three developed marginal Ab titers and another one had a CD8$^+$ T cell response to HLA-A2-restricted peptide MAGE-3 271–279. In contrast, of eight patients from the second cohort vaccinated with MAGE-3 protein and adjuvant, seven developed high-titered Abs to MAGE-3, and four had a strong concomitant CD4$^+$ T cell response to HLA-DP4-restricted peptide 243–258. One patient simultaneously developed CD8$^+$ T cells to HLA-A1-restricted peptide 168–176. The novel monitoring methodology used in this MAGE-3 study establishes that protein vaccination induces clear CD4$^+$ T cell responses that correlate with Ab production. This development provides the framework for further evaluating integrated immune responses in vaccine settings and for optimizing these responses for clinical benefit. *The Journal of Immunology,* **2004, 172: 3289–3296.**

The MAGE-3 Ag was identified during analysis of CD8$^+$ T cell reactivity against an autologous melanoma cell line and was found to be encoded by a member of a multigene family located on the X chromosome (1). MAGE-3 belongs to the growing class of cancer testis (CT)[3] Ags that are expressed only in testicular germ cells and no other normal tissue, yet aberrantly found in a broad variety of tumors (2). A major characteristic of many CT Ags is their capacity to elicit spontaneous immune reactions in cancer patients (3, 4). However, even though ~40% of nonsmall cell lung cancers express MAGE-3 (1, 2, 5, 6), patients with naturally occurring immune responses to MAGE-3 actually appear to be very rare (7–9).

Still, several CD8$^+$ T cell epitopes of MAGE-3 have been identified in vitro (10–18), including HLA-A1-restricted epitope 168–176 (1) and HLA-A2-restricted epitope 271–279 (19). Based on these findings, synthetic peptides corresponding to these epitopes have been introduced into clinical vaccination studies in which they were associated with regression of melanoma in individual cases (20). However, circulating anti-MAGE-3 CD8$^+$ T cells have been very difficult to detect, even in patients with tumor regressions (21–24).

Studies have indicated that CD4$^+$ Th cells in vivo have the capacity to enhance CD8$^+$ T cell activity (25–27) and, most importantly, help to maintain the immune response for sustained periods of time (27–29). Therefore, it seems likely that optimal antitumor activity can only be achieved if both CD4$^+$ and CD8$^+$ tumor-specific T cells are induced (30, 31). The inclusion of CD4$^+$ epitopes into MAGE-3 vaccination studies has recently been facilitated by the identification of several HLA-DR-restricted (32–35) and one HLA-DP4-restricted epitope (36, 37).

Clinical vaccination studies using full-length recombinant proteins have the advantage that this form of Ag potentially includes the full range of epitopes for CD4$^+$ and CD8$^+$ T cells. In addition, it is likely that protein vaccination leads to presentation of epitopes in the context of various HLA alleles, and therefore this type of vaccine should be applicable to any patient regardless of HLA restriction. To date, only one clinical study using MAGE-3 protein as a vaccine has been reported (38). Using a cloning approach, one patient was shown to have a CD4$^+$ T cell response to HLA-DR1-restricted peptide 267–282 (39).

We have recently introduced new methodologies for monitoring CD8$^+$ (3) and CD4$^+$ (4, 40) T cell responses in uncloned populations at the single cell level, to explore the repertoire of naturally occurring T cells against another CT Ag, NY-ESO-1. This is particularly important in the analysis of CD4$^+$ T cells, in which high background precluded the interpretation of the specificity of responses. We now applied our experience to MAGE-3 monitoring and show that vaccination with rMAGE-3 protein results in the production of anti-MAGE-3 Ab and the generation of peptide-specific CD4$^+$ and CD8$^+$ T cells in patients with nonsmall cell lung cancer.

Materials and Methods

Patients

Seventeen patients with MAGE-3-expressing stage I or II nonsmall cell lung cancer were analyzed in this study. All patients had undergone surgical resection of their primary lung tumor at the Department of Cardio-Thoracic Surgery, Weill Medical College of Cornell University, and had no

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[3] Abbreviations used in this paper: CT, cancer testis; ASC, Ag-stimulating cell; NP, nucleoprotein.

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evidence of disease at the onset of the study. Tumor expression of the gene MAGE-3 was assessed by RT-PCR. Patients provided informed consent to participate in the experimental vaccination study and to donate blood for immunological monitoring. The study was approved by the Institutional Review Board of Weill Medical College of Cornell University and sponsored by the Ludwig Institute for Cancer Research under a Ludwig Institute IND (Investigational New Drug).

Vaccine

The MAGE-3 protein preparation used in this trial was a DNA recombinant fusion protein (ProteinD MAGE-3/His) expressed in *Escherichia coli* AR58 (GlaxoSmithKline, Rixensart, Belgium), as described (38). This experimental vaccine was injected alone or in combination with adjuvant AS02B (Adjuvant System 2B; GlaxoSmithKline). Adjuvant AS02B contains monophosphoryl lipid A and QS21, a saponin extracted from the South American tree Quillaja Saponaria Molina.

Study design

The first nine consecutive patients received 300 μg of MAGE-3 protein alone; the remaining eight patients received MAGE-3 protein combined with AS02B adjuvant containing 100 μg of monophosphoryl lipid A and 100 μg of QS21 in oil/water emulsion in a final formulation volume of 500 μl. The vaccination schedule of this phase II study was as follows: four intradermal injections (protein alone cohort) or four i.m. injections (protein with adjuvant cohort) at 3-wk intervals (days 1, 22, 43, and 64). Blood for immunomonitoring purposes was drawn at five different time points (prestudy, and days 22, 43, 64, and 85).

MAGE-3 Ab

Serum IgG Ab against MAGE-3 protein was measured by ELISA using MAGE-3 full-length protein (GlaxoSmithKline) and rMAGE-3 truncated protein (aa 57–219) purified from *E. coli*, as described before (7). We used a protein from a different source than the vaccine agent, to avoid potential reactivity with protein D, and found consistent results when cotyping reactivity against the MAGE-3 protein used for vaccination. MAGE-1 and NY-ESO-1 recombinant proteins were used as negative controls, to rule out reactivity against bacterial contaminants (7). Positive results were defined from titration curves, as described before (7). In summary figures, to facilitate comparison of Ab responses between patients, all sample OD values at 1/400 serum dilution were normalized according to positive and negative control sera using the following formula: $(OD_{sample} - OD_{negative\ control})/(OD_{positive\ control} - OD_{negative\ control})$. Positive control serum from patient A25 ranged from 1000 to 1900 absorbance units, and negative control serum from patient NW29 ranged from 100 to 300 absorbance units at 1/400 dilution.

Peptides and viral vectors

MAGE-3.DP4 peptide 243–258 (KKLLTQHFVQENYLEY) was provided by Clinalfa AG (Läufelfingen, Switzerland). MAGE-3.A1 peptide 168–176 (EVDPIGHLY) and MAGE-3.A2 peptide 271–279 (FLWGPRALV) were synthesized by Multiple Peptide Systems (San Diego, CA). Influenza A nucleoprotein (NP) peptide 206–229 (FWRGENGRKTRIAYERMC NILKGK), NY-ESO-1 peptides 159–167 (LMWITQCFL), and 80–109 (ARGPESRLLEFYLAMPFATPMEAELARRSL) were obtained from Bio-Synthesis (Lewisville, TX). All peptides had a purity >90%. Vaccinia virus recombinant for full-length NY-ESO-1 was obtained from THERION Biologics (Cambridge, MA) and was constructed, as described (41). Vaccinia virus recombinant for full-length MAGE-3 was kindly provided by V. Cerundolo (Weatherall Institute of Molecular Medicine, Oxford, U.K.).

In vitro presensitization

PBMC were collected using a Ficoll gradient and were frozen in RPMI 1640 containing 10% FCS and 10% DMSO in liquid nitrogen until further processing. HLA typing of donor PBMCs or derived cell lines was done by sequence-specific oligonucleotide probing and sequence-specific priming of genomic DNA using standard procedures. $CD4^+$ and $CD8^+$ T lymphocytes were separated from PBMC of healthy donors and cancer patients using Ab-coated magnetic beads (Dynabeads; Dynal, Oslo, Norway) and seeded into round-bottom 96-well plates (Corning, NY) at a concentration of 5×10^5 cells/well in RPMI 1640 medium with 10% human AB serum (Gemini Bio-Products, Woodland, CA), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 1% nonessential amino acids. As Ag-stimulating cells (ASC) for presensitization, PBMC depleted of $CD4^+$ and $CD8^+$ T cells were pulsed with 10 μM of peptide overnight at 37°C in 500 μl of serum-free medium (X-VIVO-15; BioWhittaker, Walkersville, MD). Pulsed $CD4^-/CD8^-$ ASC were then washed, irradiated, and added to plates containing $CD4^+$ or $CD8^+$ T cells at a concentration of 1×10^6 ASC/well. After 20 h, IL-2 (10 U/ml; Roche Molecular Biochemicals, Indianapolis, IN) and IL-7 (20 ng/ml; R&D Systems, Minneapolis, MN) were added. Subsequently, one-half of medium was replaced by fresh complete medium containing IL-2 (20 U/ml) and IL-7 (40 ng/ml) twice per week.

Generation and culture of target cells

A fraction of $CD4^+$ T cells remaining from the initial separation (see above) was seeded into 24-well plates (Corning Glass, Corning, NY) at a concentration of $2–4 \times 10^6$ cells/well in complete medium supplemented with 10 μg/ml PHA (PHA HA15; Murex Diagnostics, Dartford, U.K.). Cells were fed and expanded twice per week with complete medium containing IL-2 (10 U/ml) and IL-7 (20 ng/ml). The activated T cell APCs (T-APC) were typically harvested and used as target cells after 20–30 days of culture. EBV-transformed B lymphocytes (EBV-B cells), the mutant TAP-deficient cell line T2 (CEM × 721.174.T2), and the $HLA\text{-}A1^+$/$MAGE\text{-}3^+$ melanoma cell line SK-MEL-128 were cultured in RPMI 1640 medium supplemented with 10% FCS (Summit Biotechnology, Ft. Collins, CO), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 1% nonessential amino acids. In all assays, target cell APC were washed twice in X-VIVO-15 medium to remove serum and were resuspended in appropriate medium for testing.

Tetramer staining

HLA-A1 tetramer assembled with MAGE-3.A1 peptide 168–176 (EVDPIGHLY) was a kind gift from D. Colau from the Ludwig Institute for Cancer Research (Brussels, Belgium). HLA-A2 tetramers assembled with MAGE-3.A2 peptide 271–279 (FLWGPRALV) were obtained from I. Luescher at the Ludwig Institute core facility (Lausanne, Switzerland). Presensitized $CD8^+$ T cells in 50 μl of PBS containing 3% FCS (Summit Biotechnology) were stained with PE-labeled tetramer for 15 min at 37°C before addition of Tricolor-CD8 mAb (Caltag Laboratories, South San Francisco, CA) and FITC-conjugated anti-CD62L mAb (Caltag Laboratories) or fluorescein-conjugated anti-CCR7 mAb (R&D Systems) for 15 min on ice. After washing, results were analyzed by flow cytometry (FACSCalibur; BD Biosciences, San Diego, CA).

ELISPOT assays

ELISPOT assays for the determination of Ag-specific effector cells were usually performed on day 10 of presensitizing culture for $CD8^+$ T cells and on day 20 for $CD4^+$ T cells. Flat-bottom, 96-well nitrocellulose plates (MultiScreen-HA; Millipore, Bedford, MA) were coated with IFN-γ mAb (2 μg/ml, 1-D1K; Mabtech, Stockholm, Sweden) and incubated overnight at 4°C. After washing with RPMI 1640, plates were blocked with 10% human AB-type serum for 2 h at 37°C. Target cells were pulsed at 37°C in 500 μl of serum-free medium with 10 μM peptide for 1 h (target cells for $CD8^+$ effectors) or overnight (target cells for $CD4^+$ effectors). In some assays, target cells were infected overnight with 20 PFU/cell vaccinia virus recombinant either for NY-ESO-1 or for MAGE-3. Target cells were washed twice and were resuspended in RPMI 1640 medium without serum. A total of 5×10^4 or 1×10^4 presensitized $CD4^+$ or $CD8^+$ T effector cells and 1×10^5 targets cells (T2 cells, T-APC, or EBV-B cells) was added to each well and incubated for 20 h. Plates were then washed thoroughly with water containing 0.05% Tween 20, and anti-IFN-γ mAb (0.2 μg/ml, 7-B6-1-biotin; Mabtech) was added to each well. After incubation for 2 h at 37°C, plates were washed and developed with streptavidin-alkaline phosphatase (1 μg/ml; Mabtech) for 1 h at room temperature. After washing, substrate (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium; Sigma-Aldrich, St. Louis, MO) was added and incubated for 5 min. Plate membranes displayed dark-violet spots that were scanned and counted using C.T.L. ImmunoSpot analyzer and software (Cellular Technologies, Cleveland, OH).

Measurement of intracellular cytokines (CYTOSPOT)

Pulsed T-APC were stained for 10 min at 37°C in 500 μl of X-VIVO-15 with 0.2 μM CFSE (Molecular Probes, Eugene, OR). Target cells were then washed with cold complete medium and were resuspended in X-VIVO-15. Presensitized $CD4^+$ effector T cells were incubated with peptide-pulsed $CD4^+$ T-APC at a 1:2 ratio in 200 μl of X-VIVO-15 at 37°C for 2 h. Brefeldin A (Sigma-Aldrich) at 10 μg/ml was added to each sample and cells were incubated for an additional 5-h period. Cells were then fixed using FACS Lysing Solution (BD Biosciences) diluted 1/10, permeabilized using Permeabilizing Solution 2 (BD Biosciences), and stained with Tricolor-labeled anti-CD4 mAb (Caltag Laboratories), APC-labeled anti-IFN-γ mAb, and PE-labeled anti-IL-2, anti-TNF-α, anti-IL-4, anti-IL-5,

and anti-IL-10 mAb (BD PharMingen, San Diego, CA) at room temperature for 15 min. Cells were subsequently analyzed by flow cytometry with gating on morphologically defined lymphocytes and CD4-positive and CFSE-negative cells.

Results

Vaccination with MAGE-3 protein in combination with adjuvant AS02B results in the generation of anti-MAGE-3 Abs

Of nine patients who had been vaccinated with MAGE-3 protein in the absence of adjuvant, three (WS-07, AS-08, and SG-09) developed a modest, but significant increase in Abs against MAGE-3 protein, as measured by ELISA (Fig. 1, *left*). In contrast, of eight patients who received MAGE-3 protein in combination with adjuvant AS02B, seven showed a marked increase in serum concentrations of anti-MAGE-3 (Fig. 2, *left*). Increases in Ab titers usually became significant on sample day 43, 3 wk after the patients had received the vaccine for the second time (see Fig. 3 for representative patients in titration experiments). Maximum serum levels were reached at day 85, the end of the observation period after four vaccine injections.

Vaccination with MAGE-3 protein in combination with adjuvant AS02B evokes a strong CD4$^+$ T cell response

We have previously introduced activated T cells (T-APC) as targets in a modified IFN-γ ELISPOT assay (40). In this highly sensitive assay, Ag-specific CD4$^+$ T cells can be detected on the basis of their cytokine secretion over a very low background. Using the same technique, we analyzed CD4$^+$ T cell responses against MAGE-3 in all patients.

To examine whether these patients had in principle the capacity to develop a CD4$^+$ T cell response, or whether T cell immunity might have been compromised by malignant disease, we first examined CD4$^+$ T cell responses directed against a promiscuous epitope of influenza NP. All patients, with the exception of DS-03, showed good responses (mean: 399 spots/50,000 CD4$^+$ T cells) against T-APC pulsed with NP peptide 206–229 (data not shown).

We then examined CD4$^+$ T cell responses against peptide MAGE-3.DP4 in all 17 patients. This epitope was chosen for the frequent distribution of its restriction allele HLA-DP4 and its proven immunogenicity (37). We observed that only one of the patients who had received MAGE-3 protein without adjuvant AS02B showed a CD4$^+$ T cell response against MAGE-3.DP4 in ELISPOT assays (Fig. 1, *middle*). It seemed that in this patient (ST-04) expressing the HLA-DP4 haplotype (Table I), a pre-existing immunity against MAGE-3 was present, because his CD4$^+$ T cells secreted IFN-γ in response to MAGE-3.DP4-pulsed T-APC even before he had received the first vaccination. Also, the same patient had low-titered pre-existing Ab titers against MAGE-3 protein. However, repeated immunizations with MAGE-3 protein in the absence of AS02B adjuvant did not have a boosting effect on the level of MAGE-3.DP4-specific T cell immunity or the level of anti-MAGE-3 Abs in this specific patient.

All eight patients who had received MAGE-3 protein in combination with adjuvant AS02B were found to have the HLA-DP4 allele (Table I). Of these patients, four (WG-13, ER-14, GT-15, and GO-17) showed a marked increase in CD4$^+$ T cell responses against MAGE-3.DP4 (Fig. 2, *middle*). All responding patients had

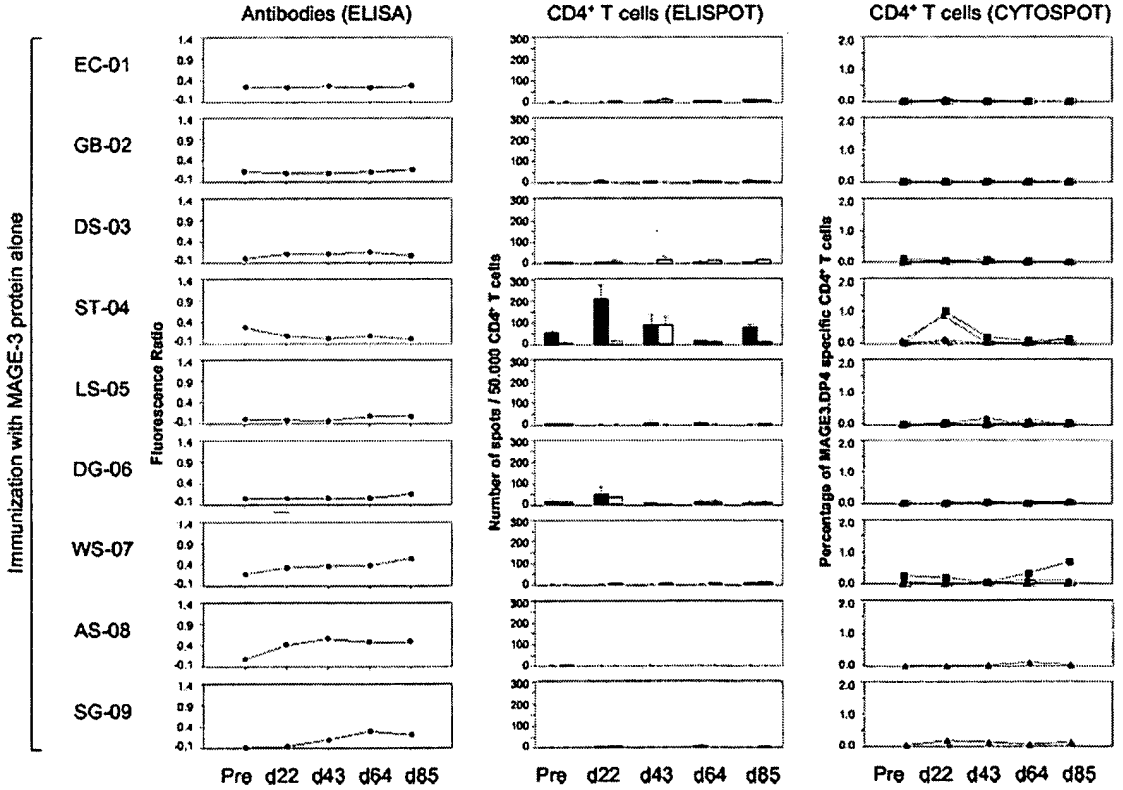

FIGURE 1. Monitoring of Ab and CD4$^+$ T cell responses in patients vaccinated with MAGE-3 protein without adjuvant. *Left*, Ab responses to MAGE-3 by ELISA at different vaccination time points. To facilitate comparison between patients, all sample OD values at 1/400 serum dilution were normalized according to positive and negative controls. *Middle*, CD4$^+$ T cell responses as measured by IFN-γ ELISPOT. Presensitized CD4$^+$ T cells (50,000) were tested against T-APC pulsed with MAGE-3.DP4 (■) or irrelevant peptide (□). *Right*, CD4$^+$ T cell responses as measured by staining of intracellular cytokines (CYTOSPOT). Percentage of presensitized CD4$^+$ T cells specifically producing TNF-α (■), IFN-γ (▲), IL-2 (●), IL-10 (□), IL-4 (△), or IL-5 (○) against MAGE-3.DP4.

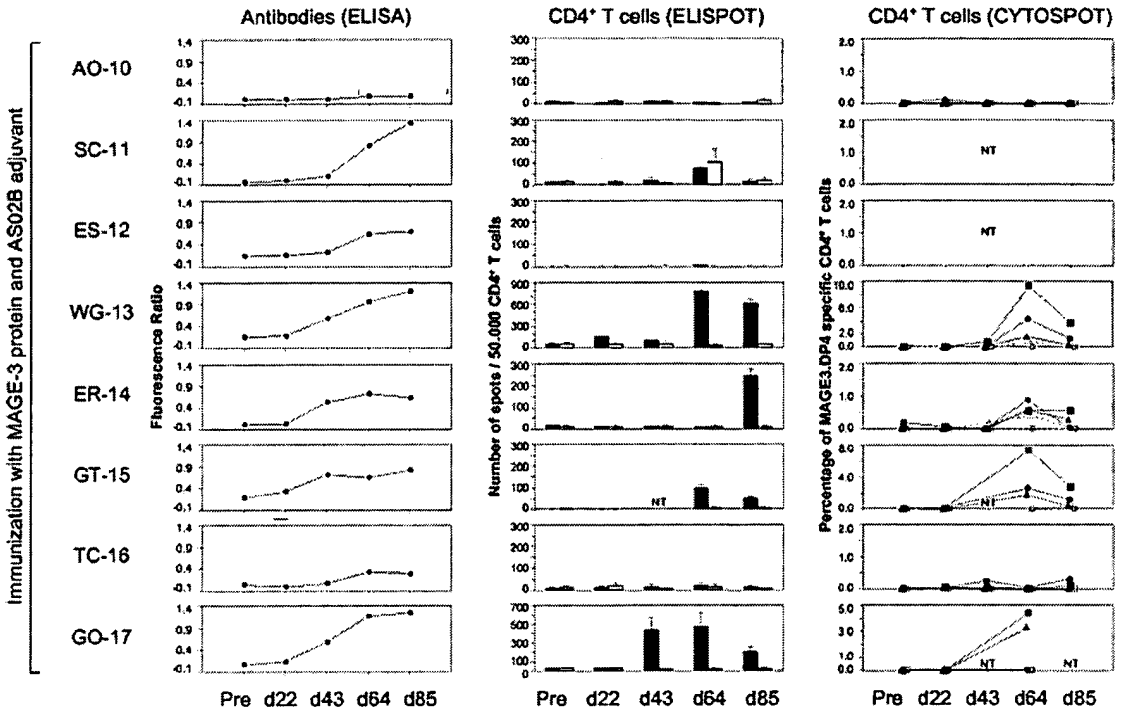

FIGURE 2. Induction of strong Ab and $CD4^+$ T cell responses in patients vaccinated with MAGE-3 protein plus adjuvant. See Fig. 1 for legends.

a concomitant humoral response against MAGE-3. $CD4^+$ T cells specific for the tumor Ag appeared between days 43 and 64 and were always clearly detectable until day 85, the last sampling date. A presensitization with the irrelevant peptide NY-ESO-1 80–109 as a specificity control did not lead to the generation of effector cells specific for this epitope in any of the patients (data not shown). Numbers of IFN-γ-producing spots after presensitization with positive control peptide NP 206–229 were comparable to number of spots for MAGE-3.DP4 in responding patients (mean = 866 spots for NP/50,000 $CD4^+$ T cells for patient WG-13; 921 spots for ER-14; 71 spots for GT-15; 733 spots for GO-17).

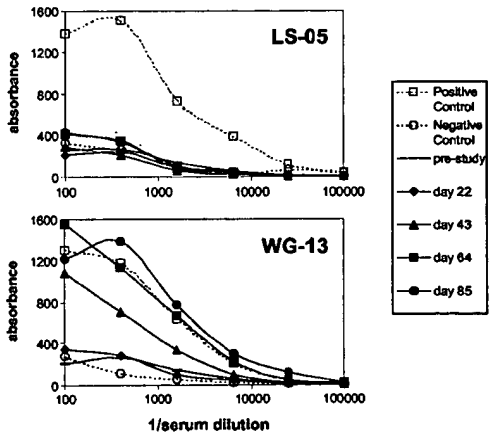

FIGURE 3. Characteristic titration curves by ELISA of patient sera responding (WG-13) or not (LS-05) to MAGE-3 protein at various study dates. Positive control serum (□) was from melanoma patient A25 with spontaneous humoral response to MAGE-3, and negative control serum (○) was from patient NW29.

$CD4^+$ T cells induced by vaccination with MAGE-3 protein in combination with adjuvant AS02B produce Th1-, but no Th2-type cytokines

We next performed a more detailed analysis of the pattern of cytokines produced by patients' $CD4^+$ T cells in response to MAGE-3.DP4. We had previously determined that our in vitro culture conditions were likely to reflect the pre-existing cytokine profile of effectors (40). Using T-APC as targets, we performed a flow cytometry analysis of a variety of intracellular Th1-type (IFN-γ, IL-2, TNF-α) or Th2-type (IL-4, IL-5, IL-10) cytokines in all but two patients (Figs. 1 and 2, *right*).

Table I. *HLA type of all patients included into the MAGE-3 protein vaccination study*[a]

	MHC Class I HLA-A		MHC Class II HLA-DP4 status
EC-01	0301	**0205**	−
GB-02	**0101**	0301	+
DS-03	2402	2402	+
ST-04	**0205**	2301	+
LS-05	0301	2402	+
DG-06	**0201**	3001	+
WS-07	**0201**	3001	−
AS-08	1101	3101	+
SG-09	**0201**	**0201**	−
AO-10	**0101**	0304	+
SC-11	**0201**	2301	+
ES-12	**0201**	0301	+
WG-13	**0101**	2501	+
ER-14	2301	3101	+
GT-15	**0101**	**0201**	+
TC-16	**0101**	0301	+
GO-17	**0201**	6801	+

[a] Shown are the HLA-A alleles and the status of HLA-DP4 expression by the given patient. Bold characters highlight patients with HLA-A1 or HLA-A2 expression.

We found that results in the ELISPOT assay were closely paralleled by those observed after staining of intracellular cytokines. Of the eight patients who received MAGE-3 protein in combination with adjuvant, the same four patients that had clear $CD4^+$ responses in the ELISPOT assays also showed MAGE-3.DP4 peptide-induced increase in intracellular cytokines (Fig. 2).

The cytokines that were produced in response to MAGE-3.DP4 Ag were almost exclusively of Th1 type. None of the patients showed significant increases in the intracellular concentrations of IL-4, IL-5, or IL-10 in response to MAGE-3. In contrast, we observed marked increases in the intracellular concentration of IFN-γ and IL-2 following exposure to T-APC pulsed with the MAGE-3.DP4 peptide. TNF-α, however, seemed to be by far the most sensitive parameter for the detection of MAGE-3-specific $CD4^+$ T cells, as shown in a representative patient (Fig. 4).

Vaccination with MAGE-3 protein induces MAGE-3.A1- and MAGE-3.A2-specific $CD8^+$ T cells

We looked for the presence of MAGE-3.A1- or MAGE-3.A2-specific T cells in patients that had been vaccinated with MAGE-3 protein by performing tetramer analyses (Table II) and ELISPOT assays (Table III).

Of nine $HLA\text{-}A2^+$ patients analyzed, one (DG-06) showed a significant increase in tetramer-positive MAGE-3.A2-specific $CD8^+$ T cells on day 85. These T cells were negative for CD62L and CCR7, and therefore expressed an effector phenotype (Fig. 5*A*). When an ELISPOT assay was performed using these effector cells, they recognized T2 cells or T-APC pulsed with the HLA-A2-restricted epitope of MAGE-3 (Fig. 5*B*).

Of five $HLA\text{-}A1^+$ patients analyzed, one (GT-15) showed a marked increase in tetramer-positive MAGE-3.A1-specific $CD8^+$ T cells from day 43 onward. These T cells expressed intermediate levels of CD62L and were negative for CCR7 (Fig. 6*A*). However, an IFN-γ ELISPOT assay performed using this effector cell line revealed that these $CD8^+$ T cells did not recognize EBV-B cells, whether they were pulsed with MAGE-3.A1 peptide or infected with vaccinia virus recombinant for MAGE-3. Furthermore, the same $CD8^+$ T cells did not recognize a MAGE-3-expressing $HLA\text{-}A1^+$ melanoma cell line even after this tumor cell line had been pulsed with MAGE-3.A1 peptide (Fig. 6*B*).

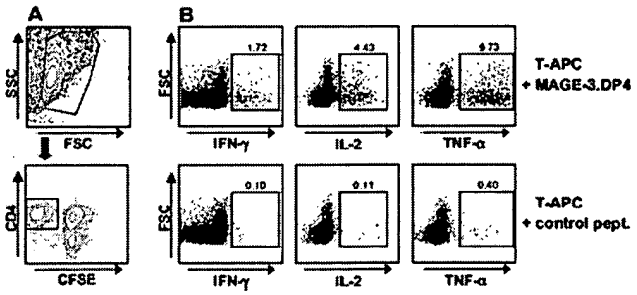

FIGURE 4. Vaccination with MAGE-3 protein induces a strict Th1 type of response against peptide MAGE-3.DP4. The figure shows the typical pattern of intracellular cytokine staining in a patient (WG-13) responding to the vaccine. PBL were obtained on day 64 after study onset, and MAGE-3.DP4-specific $CD4^+$ T cells were expanded in a single cycle of peptide-driven stimulation. FACS analysis of intracellular concentration of Th1-type (IFN-γ, IL-2, TNF-α) and Th2-type (IL-4, IL-5, IL-10) cytokines was performed on day 25 postculture initiation. To distinguish them from effector cells, $CD4^+$ T-APC were stained using the intracellular dye CFSE before the assay was started. Targets were later excluded from the analysis using double gating on morphologically defined lymphocytes and gating on CFSE-negative and $CD4^+$ cells. Responding patients usually showed strong Th1-type responses against peptide MAGE-3.DP4-pulsed T-APC. Th2-type cytokine concentration did not exceed background levels (data not shown). Background levels were determined using T-APC pulsed with the irrelevant control NY-ESO-1 peptide 80–109 and were typically below 0.1% of effector $CD4^+$ T cells.

Table II. *$CD8^+$ responses against MAGE-3 as indicated by tetramer analysis*[a]

		Pre	Day 22	Day 43	Day 64	Day 85
MAGE-3.A1	GB-02	0.09	0.07	0.04	0.06	0.03
	LS-05	0.08	0.08	0.08	0.05	0.14
	AO-10	0.10	0.11	0.05	0.07	0.10
	WG-13	0.23	0.14	0.12	0.13	0.14
	GT-15	0.31	0.11	**3.23**	**6.59**	**0.79**
	TC-16	0.14	0.14	0.09	0.1	0.08
MAGE-3.A2	EC-01	0.00	0.02	0.00	0.00	0.02
	DS-03	0.01	0.01	0.01	0.01	0.00
	DG-06	0.10	0.05	0.14	0.16	**1.60**
	WS-07	0.10	0.00	0.06	0.00	0.03
	SG-09	0.06	0.00	0.01	0.19	0.10
	SC-11	0.00	0.05	0.03	0.02	0.00
	ES-12	0.08	0.14	0.07	0.12	0.07
	GT-15	0.00	0.00	0.00	0.04	0.01

[a] All five $HLA\text{-}A1^+$ patients and eight $HLA\text{-}A2^+$ patients were analyzed $CD8^+$ T cells were expanded by a single peptide-driven stimulation with peptide MAGE-3.A1 (168–176) or MAGE-3.A2 (271–279), respectively. Effector cells were tested on day 10 against using the given tetramer. Numbers given indicate the percentage of tetramer-positive $CD8^+$ T cells. Bold numbers represent significant positive responses.

Discussion

Successful vaccination of animals with tumor Ags has been shown to be largely dependent on $CD8^+$ T cells, and adoptive transfer of purified $CD8^+$ T cell fractions can mediate tumor regression in mice (42). However, it has recently become clear that $CD4^+$ T cells play an important role in the antitumor response following vaccination (43, 44). Although $CD4^+$ T cells seem to also possess the potential for an immediate effector function against tumor targets, in vitro (45–47) and in vivo (48), their main role is still widely believed to be that of a helper cell. Thus, activated $CD4^+$ T cells help to initiate, amplify, and maintain $CD8^+$ T cell responses. They do so by providing important costimulation via a variety of surface molecules (49), by secretion of a network of cytokines (50), and by activating professional APC (51–56). In addition, studies have suggested that $CD4^+$ T cells have the capability to recruit eosinophils as well as macrophages into the tumor tissue. These cells, activated by neighboring tumor-specific $CD4^+$ T cells, might then contribute to an effective antitumor function by producing factors such as superoxide and NO (57).

We have used a rMAGE-3 protein as a vaccine in patients with nonsmall cell lung cancer. This approach, in addition to the generation of MAGE-3-specific $CD8^+$ T cells, theoretically allows the introduction of specific $CD4^+$ T cell help into the T cell-mediated antitumor reaction. We show in this study that vaccination with a protein of a CT Ag indeed provides a strong peptide-specific $CD4^+$ T cell response. The occurrence of $CD4^+$ T cell responses correlated with Ab responses. Importantly, we monitored this protein-based vaccination study using peptides as Ag in our assays. This excludes the possibility of T cell responses against contaminants in the protein batch that have been observed in assays using MAGE-3 protein as stimulating Ag and in the readout assay (32).

The MAGE-3 protein-induced $CD4^+$ T cell responses were of the Th1 type, suggesting a supporting role of these Th cells for anti-MAGE-3 $CD8^+$ T cell responses in vivo. In addition, it has previously been shown that MAGE-3.DP4-specific $CD4^+$ T are in principle capable of lysing $HLA\text{-}DP4^+$ tumor cells expressing

Table III. *$CD8^+$ responses against MAGE-3, as indicated by ELISPOT analysis*[a]

		Pre	Day 22	Day 43	Day 64	Day 85
MAGE-3.A1	GB-02	113 (113)	28 (26)	36 (50)	32 (26)	3 (3)
	AO-10	45 (38)	38 (36)	38 (39)	28 (25)	44 (40)
	WG-13	106 (123)	65 (89)	106 (74)	128 (111)	99 (89)
	GT-15	18 (15)	11 (3)	15 (22)	54 (53)	31 (45)
	TC-16	165 (174)	139 (144)	115 (109)	158 (181)	129 (162)
MAGE-3.A2	EC-01	12 (0)	7 (5)	3 (7)	6 (4)	13 (4)
	DS-03	0 (1)	1 (0)	0 (3)	0 (0)	0 (1)
	DG-06	67 (18)	11 (13)	2 (0)	139 (109)	**214** (53)
	WS-07	3 (2)	2 (2)	4 (0)	7 (0)	4 (9)
	SG-09	9 (2)	9 (8)	10 (8)	23 (5)	5 (2)
	SC-11	3 (6)	1 (6)	3 (5)	1 (1)	4 (14)
	ES-12	28 (62)	11 (18)	2 (11)	18 (12)	17 (13)
	GT-15	6 (3)	3 (0)	0 (4)	2 (0)	1 (8)
	GO-17	4 (16)	4 (5)	8 (54)	8 (3)	16 (14)

[a] All five HLA-$A1^+$ patients and nine HLA-$A201^+$ patients were analyzed. $CD8^+$ T cells were expanded by a single peptide-driven stimulation with peptide MAGE-3.A1 (168–176) or MAGE-3.A2 (271–279), respectively. Effector cells were tested on day 10 against the HLA-$A1^+$ EBV-transformed B cell line from healthy donor NC 32 or T2 cells, respectively. Targets were pulsed with the relevant MAGE-3 peptide. Numbers given indicate specific IFN-γ spots per 50,000 $CD8^+$ effector T cells. Numbers in brackets indicate background reactivity against unpulsed targets (for HLA-$A1^+$ patients) or targets pulsed with the irrelevant NY-ESO-1 peptide 159–167 (for HLA-A*0201^+ patients). Bold numbers represent significant positive responses.

MAGE-3 (36), indicating that this epitope, in contrast to other class II MAGE-3 epitopes (32), may be presented on the surface of tumor cells.

In addition to the strong $CD4^+$ T cell responses in 50% of patients receiving MAGE-3 protein plus adjuvant, we also observed peptide-specific $CD8^+$ T cell responses in two patients. Previous in vitro studies have indicated that the HLA-A1-restricted MAGE-3 peptide 168–176 is naturally processed by professional (58) and nonprofessional APC (1, 59), and the same peptide has been eluted from cancer cell lines (60). In this study, we show that the MAGE-3.A1 peptide is naturally processed in vivo and that this leads to a strong increase in peripheral numbers of $CD8^+$ T cells specific for this epitope, in coordination with MAGE-3 $CD4^+$ T cell and Ab responses. The vaccine-induced MAGE-3.A1-specific $CD8^+$ T cells could easily be expanded using a single cycle of peptide-driven stimulation. De novo $CD8^+$ T cells were not generated during this in vitro expansion phase because reactivity to MAGE-3.A1 was not detected before the second vaccination. Rather, $CD8^+$ T cells appeared as a result of vaccination, concomitantly to Ab and $CD4^+$ T cell responses in an individual patient. Following this 10-day culture period, the MAGE-3.A1 tetramer-positive cells expressed an effector cell phenotype. However, these $CD8^+$ T cells did not produce IFN-γ in response to target cells pulsed with MAGE-3.A1 peptide or MAGE-3-expressing HLA-$A1^+$ tumor cells. We did not examine whether the MAGE-3.A1-specific T cells produced other cytokines (i.e., T cytotoxic type 2 cytokines) in response to their respective epitope, and it remains to be further examined whether these cells have to be considered partially nonresponsive. MAGE-3.A1-specific $CD8^+$ T cells have previously been shown to kill MAGE-3-expressing tumor cells (1, 59), and future vaccination studies will

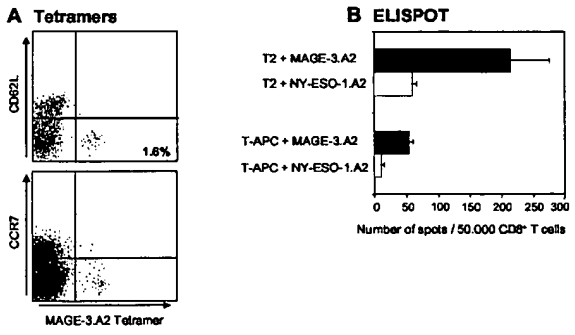

FIGURE 5. MAGE-3 protein vaccination-induced generation of fully functional MAGE-3.A2-specific $CD8^+$ T cells. PBL of patient DG-06 were obtained on day 85 after study onset, and MAGE-3.A2-specific $CD8^+$ T cells were expanded in a single cycle of peptide-driven stimulation. Tetramer analysis (*A*) was performed on day 10 postculture initiation. The $CD8^+$ T cells were costained for CD62L and CCR7. An ELISPOT assay (*B*) was performed on the same day using this tetramer-positive $CD8^+$ T cell line. Effector cells were tested against T2 cells or T-APC pulsed with MAGE-3.A2 peptide or the irrelevant NY-ESO-1 peptide 159–167.

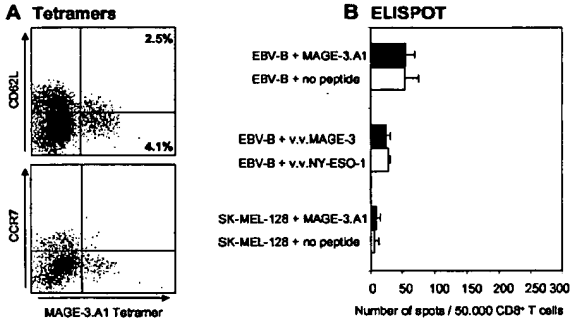

FIGURE 6. MAGE-3 protein vaccination-induced generation of non-IFN-secreting MAGE-3.A1-specific $CD8^+$ T cells. PBL of patient GT-15 were obtained on day 64 after study onset, and MAGE-3.A1-specific $CD8^+$ T cells were expanded in a single cycle of peptide-driven stimulation. Tetramer analysis (*A*) was performed on day 10 postculture initiation. The $CD8^+$ T cells were costained for CD62L and CCR7. An ELISPOT assay (*B*) was performed on the same day using this tetramer-positive $CD8^+$ T cell line. Effector cells were tested against autologous EBV-transformed B cells unpulsed or pulsed with MAGE-3.A1 peptide. The same tetramer-positive line was also tested against autologous EBV-B cells infected with vaccinia virus recombinant for full-length MAGE-3 or recombinant for NY-ESO-1 as irrelevant Ag. Finally, reactivity of the MAGE-3.A1-specific $CD8^+$ T cell line was tested against the peptide-pulsed or unpulsed HLA-$A1^+$, MAGE-3-expressing melanoma cell line SK-MEL-128.

show whether stronger adjuvants delivered with MAGE-3 protein or more prolonged immunization might lead to the in vivo generation of fully functional T cytotoxic type 1 MAGE-3.A1-specific CTL.

In vitro studies have indicated that the HLA-A2-restricted MAGE-3 epitope 271–279 is not naturally processed by nonprofessional APC, including most tumor cells (61). This seems to be caused by cleavage of the MAGE-3 protein at position 278 during its processing by the proteasome (62, 63). In contrast, it has been shown that professional APC using the immunoproteasome are capable of generating antigenic MAGE-3 peptides that are not produced by a standard proteasome (18), possibly including MAGE-3.A2. Accordingly, it has been shown that the processing of MAGE-3-expressing tumor cells by dendritic cells may result in the generation of $CD8^+$ T cells specific for peptide MAGE-3 271–279 (64–66).

We observed that the MAGE-3.A2 epitope seems to be naturally processed in vivo because the vaccination with rMAGE-3 protein resulted in the appearance of MAGE-3.A2-specific $CD8^+$ T cells in the peripheral blood of one patient. These $CD8^+$ T cells could easily be expanded using a single peptide-driven stimulation and were only detected after vaccination. Although MAGE-3.A2-specific $CD8^+$ T cells were seen in the absence of $CD4^+$ T cell or Ab response to MAGE-3, they were fully functional in the sense that they secreted IFN-γ in response to target pulsed with their respective peptide. Therefore, we suggest that in vivo MAGE-3 protein might have been taken up and processed in patients by professional APC, resulting in the generation of $CD8^+$ T cells specific for MAGE-3.A2 peptide 271–279. It remains questionable, however, whether these T cells will have clinical efficacy against nonprofessional APC, such as autologous tumor cells, even if these cells express the MAGE-3 gene.

In conclusion, we show in this study that vaccination with the recombinant protein of a CT Ag provides strong Ag-specific $CD4^+$ T cell help along with Ab and $CD8^+$ T cell responses, and leads to integrated immunity comparable to what is observed in patients with spontaneous responses to NY-ESO-1 (4). It is likely that responses against a range of $CD4^+$ and $CD8^+$ epitopes other than the ones we examined in this study were generated, and application of general methodologies (40, 41) will allow the identification of this repertoire.

The current study design included patients with no evidence of disease at the onset of the trial, precluding assessment of clinical efficacy at this early stage. The data presented in this work lay the grounds for the design of vaccine constructs and immunization protocols to define conditions for maximal immunogenicity and answer the most important question in tumor immunology: can immunization affect the course of human cancer?

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Activated Akt Is Frequently Overexpressed in Human Breast Cancer Cells and Its Blockade Can Inhibit Growth. *A. Al-Janadi, S. Ramachandran, N. Kuwajerwala, M. Huang, R. Chapman, B. W. Yu; Josephine Ford Cancer Ctr, Henry Ford Health System, Detroit, MI*

Akt (or protein kinase B, PKB) is a serine-threonine kinase that is activated by insulin or IGF-1 receptor signaling in a PI3-kinase dependent manner, and is implicated in breast, ovarian, pancreatic and prostate cancers. The phosphorylation of its substrate proteins result in increased growth and decreased susceptibility to apoptosis. A survey for total Akt protein expression in 11 human breast cancer cell lines cultured in media with serum, showed that 9 of the 11 have significant expression, compared to low or absent levels in nontransformed 184B5, MCF-10A, or normal human mammary epithelial cells. Moreover, some degree of Akt activation (assayed by immunoblotting with an anti-phospho-Akt (Ser473) antibody) was detected in 6 of the 11 breast cancer cell lines. Five of the 11 breast cancer cell lines were cultured in serum free media with or without insulin. The in vitro growth of four cells lines with moderate or high relative levels of activated Akt (SKBR3, BT474, BT549 and BT20) were relatively growth independent of insulin. However, the MCF-7 cell line, with minimally activated Akt grew four-fold in response to insulin. The BT-20 cell line which has highly activated Akt levels (and lacks alteration in the PTEN phosphatase), was liposomally transfected with a dominant-negative mutant human Akt1 cDNA (DN-Akt) to determine if activated Akt was required for growth in media with serum. Preliminary results show that transient transfection with DN-Akt significantly reduced in vitro growth of BT-20 cells compared to control plasmid. While 50 uM PD 098059 (a MEK inhibitor) was minimally inhibitory, combining it with the DN-Akt transfection resulted in more growth inhibition of BT-20 cells than either agent alone. These results suggest that 1. Activated Akt is often overexpressed in human breast cancer cells and may be associated with less dependence on insulin dependent signaling; and 2. Inhibition of Akt signaling may inhibit growth of breast cancer cells with high levels of activated Akt, especially in combination with blockade of other signal pathways. Akt may be a worthwhile target for intervention in human breast cancer.

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Prodrug Enzyme-Activation Cancer Gene-Therapy Using Methioninase and Selenomethionine. *K. Miki, A. Gupta, W. Al-Refaie, M. Xu, Y. Tan, M. Bouvet, T. Chishima, H. Shimada, M. Makuuchi, A. R. Moossa, R. M. Hoffman; AntiCancer, Inc, San Diego, CA; Univ of CA, San Diego, CA; Yokohama City Univ, Yokohama, Japan; Univ of Tokyo, Japan*

A novel enzyme-activation prodrug gene therapy strategy using the methionine α,γ-lyase gene (MET) cloned from *Pseudomonas putida* and selenomethionine (SeMET) as a pro-drug has been developed. We have constructed recombinant adenovirus vectors encoding the MET gene driven by the CMV-5 or CEA promoter (rAd-MET). The combination of MET gene transfer and SeMET converts the physiological compound SeMET to toxic methylselenol. The IC_{50} of SeMET on A549 lung cancer cells transduced with rAd-MET was 0.5 μM, which was 400 times less than that of non-transduced cancer cells or cancer cells transduced with control adenovirus. The combination of rAd-MET and SeMET was active against all cancer cell types tested, including head and neck, pancreatic, ovarian and lung cancer. Non-transduced cancer cells were co-cultured with rAd-MET transduced cells in various ratios to determine the bystander effect. The administration of SeMET to cultures containing only 3% rAd-MET-transduced cells resulted in the death of more than 80% of the non-transduced cells. This strong bystander effect was due to the release of toxic methylselenol from the cancer cells transduced with the MET gene. High levels of superoxide were produced in the rAd-MET transduced cells in the presence of SeMET. Apoptosis occurred in the rAdMET/SeMET-treated cancer cells as determined by nucleosomal DNA fragmentation, flow cytometry and the TUNEL assay. The apoptosis may be due to superoxide production. The CEA promoter has enabled a 10-fold selectivity for CEA-expressing cells compared to non-CEA-expressing cells. The METgene-SeMET strategy differs from other current approaches to cancer gene therapy and has potential for broad application.

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Improved MDR-1 Gene Transfer: Implications for Successful Gene Therapy with Retroviral Vectors. *A. J. Schilz, B. Schiedlmeier, S. Frühauf, C. Baum, A. A. Fauser, K. Kühlcke, H. Eckert; EUFETS GmbH, Idar-Oberstein, Germany; German Cancer Ctr, Heidelberg, Germany; Heinrich-Pette-Institut, Hamburg, Germany; Clin for BMT and Hematology/Oncology, Idar-Oberstein, Germany*

MDR-1 gene transfer into hematopoietic stem cells is an attractive approach to protect hematopoietic cells from dose-limiting side effects of posttransplantation chemotherapy and to select gene modified cells in vivo. However, clinical trials, so far, have failed to show the applicability of such an approach. Main reasons for this might be low transduction efficiencies of hematopoietic stem and early progenitor cells with retroviral vectors and an insufficient expression of the MDR-1 cDNA in the target cells. In an effort to overcome these limitations we developed optimized methods that may justify more promising clinical studies. These improvements include producer cell establishment, retroviral vector production as well as transduction into human CD34+ cells in clinical relevant scales. Using FMEV-type vectors designed for high expression in hematopoietic stem and myeloid progenitor cells and our optimized conditions for vector production on multi-tray cell factories (NUNC), we routinely obtain vector supernatants with titers of several x 10E6 vector particles/ml in serum-free medium. These supernatants were used to optimize transduction procedures for human CD34+ cells with respect to cell densities, cytokines and colocalization strategies. After obtaining satisfactory results in small scale assays, we transduced CD34+ enriched PBSC in a clinical relevant scale (~10E8 cells). Transduction efficiencies as assessed by MDR-1 functional in vitro assays were up to 50 %. The repopulating potential of the transduced cells was tested in NOD/SCID mice. Using real time quantitative PCR we found > 30 % vector-transduced human cells 8 to 10 weeks after transplantation. We expect that the presented findings will help to demonstrate that myeloprotection through MDR-1 gene transfer is feasible.

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Phase I/II Study of Vaccination with a MAGE-3 Protein Plus Immunological Adjuvant SB AS-2 in HLA Class I Selected Patients with MAGE-3 Positive Tumors. *W. H. Kruit, N. van Baren, Y. Humblet, C. J. Punt, M. Avril, B. Escudier, S. Aamdal, A. M. Eggermont, L. Hakansson, U. Keilholz, F. Lehmann, W. Gerritsen, M. Gueguen, J. Wanders, J. Uiters, M. Delire, G. Stoter, M. Marchand; Univ Hosp Rotterdam for the EORTC and NDDO Oncology, Rotterdam, Netherlands; SmithKline Beecham Biologicals, Belgium; Ludwig Institute for Cancer Research, Brussels, Belgium*

The MAGE gene family is expressed in a variety of malignancies, but not in normal tissues except for testis. Hence, MAGE-encoded antigens are tumor-specific potential targets for vaccine therapy. In this phase I/II study patients with metastatic MAGE-3 positive solid tumors were vaccinated with MAGE-3 protein with or without the adjuvant SB AS-2 to evaluate toxicity and response. Eligible patients were HLA A1, A2 or B44 positive, had WHO performance status of 0–1, normal hematologic, cardiopulmonary, renal and hepatic function. Vaccinations were given intramuscularly on day 1, 22, 43 and 64. Patients without progression received 2 additional injections on day 106 and 148. Fifty-seven patients (5, 21, 13, and 18 in 4 respective groups) were enrolled: 49 melanoma, 3 bladder, 2 non-small cell lung, 2 esophageal and 1 head and neck cancer. The first group received MAGE-3 only (300 μg). Dose levels of MAGE-3 in the other 3 groups were 30, 100, and 300 μg in combination with a fixed dose of 100 μg SB AS-2. Treatment was well tolerated with transient mainly grade I and II toxicities, with no apparent dose-related differences. Most commonly seen were tenderness and redness at the injection site, fatigue, fever, flu-like symptoms, myalgia and nausea. Grade III myalgia and local toxicity were observed in 2 patients each. Hematologic toxicity was not observed. In 36 patients who received at least 4 vaccinations, 7 clinical responses were noted with two partial responses (melanoma, bladder), 3 mixed responses (melanoma) and 2 stable diseases (melanoma), for a duration of 4–12 months. The majority of patients receiving vaccine plus adjuvant developed a MAGE-3 antibody response. No clear correlation existed between clinical and antibody responses. In conclusion, immunization with MAGE-3 protein and the adjuvant SB AS-2 was well tolerated and induced beneficial antitumor responses in a number of patients.